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INVESTIGATIONS CONCERNING HYDROLYSIS AND STABILIZATION OF ANTIRADIATION COMPOUNDS

ANNUAL REPORT

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January 1985

Supported by
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701-5012

Contract DAMD17-80-C-0128

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188
Exp. Date: Jun 30, 1986

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Distribution limited to U.S. government agencies only; contractor performance evaluation; January 1985
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Southwest Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) 6220 Culebra Road P.O. Drawer 28510 San Antonio, Texas 78284	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-80-C-0128
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62734A; PROJECT NO. 3M162, 734A875; TASK NO. AK; WORK UNIT ACCESSION NO. 089

11. TITLE (Include Security Classification)
(U) Investigations Concerning Hydrolysis and Stabilization of Antiradiation Compounds

12. PERSONAL AUTHOR(S)
D.J. Mangold, N.F. Swynnerton, C.W. Lew, E.P. McGovern, T.M. Ludden, P. Steele

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 1/15/84 TO 1/4/85	January 1985	173

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	03	
06	13	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Antiradiation compounds: ethiofos, WR-2721, WR-1065, WR-33278, hydrolytic and thermal properties, (see back) over

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

This report contains results of the following studies:

1. Encapsulation of ethiofos for use as oral dosage forms;
2. In vitro evaluation of the most promising samples;
3. Development of analytical methodology for ethiofos, WR-1065, WR-33278 and
4. Pharmacokinetic results of in vivo studies.

(keywords:

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Judy Pawlus	22b. TELEPHONE (Include Area Code) 301-663-7325 22c. OFFICE SYMBOL SGRD-RMI-S

DD FORM 1473, 84 MAR

83 APR edition may be used until exhausted.
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SECURITY CLASSIFICATION OF THIS PAGE

Block 18. (Continued)

cont'd

encapsulation, drug dosage forms, blood plasma assay, pharmacokinetics

FOREWORD

Citations of organizations and trade names do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUMMARY

This report covers studies performed under Contract 17-80-C-0128 during the period January 14, 1984 through January 4, 1985 which includes portions of the fourth and fifth year of the contract. The most significant results obtained during this period are the following.

(1) The HPLC ethiofos plasma assay was modified to give greater selectivity for unchanged drug in animal dosing studies.

(2) Pharmacokinetic parameters were determined from data generated using the improved ethiofos HPLC analytical method.

(3) An assay for the metabolite WR 1065 in plasma was developed and characterized.

(4) Pharmacokinetic modeling was carried out using the WR 1065 assay method.

(5) An analytical procedure for WR 33278, the disulfide of WR 1065, was developed and two preliminary dosings of beagle dogs with the drug were completed. Analysis of plasma samples from one of the experiments has been completed.

(6) Work on a procedure to determine covalently bound WR 1065 has begun and initial results on actual dosing samples are encouraging.

(7) The stability of ethiofos in plasma samples stored at -20°C and at -75°C for up to six months has been determined. Both the relative stability (to an internal standard) and "absolute" stability (to an external standard) were measured.

(8) The rhesus monkey was used in twelve dosing experiments and found to be much less susceptable than the beagle dog to vomiting during the experiments.

(9) Formulations containing ethiofos were prepared, evaluated in vitro and administered orally to beagle dogs and rhesus monkeys. Low concentration of ethiofos and of unbound WR 1065 were measured post administration. Analysis of plasma samples for "bound" WR 1065 are in progress.

(10) Ethiofos was administered to a beagle dog via a two-hour infusion. Plasma ethiofos levels were measured during the infusion and post infusion. Maximum concentration was 92 µg/mL with a rapid decline beginning at time infusion ended.

(11) Study Reports 5 and 6 were prepared and submitted which gave details of the assay procedures and pharmacokinetics of ethiofos and WR 1065, respectively.

(12) A paper entitled "An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma" was published in the International Journal of Radiation Oncology·Biology·Physics, Vol. 10, pps 1521-1524 (1984). Authors were N.F. Swynnerton, E.P. McGovern, J.A. Nino and D.J. Mangold.

(13) A paper entitled "HPLC Assay for 2-(3-Aminopropylamino)-ethanethiol was published in the same journal, Vol. 10, pps 1517-1520 (1984). Authors were E.P. McGovern, N.F. Swynnerton, P.D. Steele and D.J. Mangold.

(14) Three abstracts of papers concerning analysis and pharmacokinetics of ethiofos, WR 1065 and WR 33278 were submitted for presentation at the meeting of the Radiation Research Society in May, 1985 at Los Angeles, California.

I. STATEMENT OF PROBLEM

Over a period of several years, the U.S. Army Medical Research and Development Command has been actively pursuing the development of a drug or combination of drugs which could be taken by military personnel for protection from the effects of the ionizing radiations from a nuclear weapons attack. Several chemical compounds were found to be promising in animal studies, in particular the phosphorothioates. When administered intravenously the best of these materials, S-2-(3-aminopropylamino)ethyl phosphorothioic acid, ethiofos (WR 2721), has been shown to protect mice, dogs, and rhesus monkeys against X-ray and/or gamma radiation and to protect mice against neutron radiation. However, oral dosing of ethiofos failed to protect either dogs or monkeys and produced vomiting in dogs.

In an attempt to explain the lack of activity following oral administration, it has been postulated that ethiofos is readily hydrolyzed to the thiol in the stomach of the animal species and that the thiol is poorly absorbed. (Credence has been given to this hypothesis as a result of studies completed during the first year of this program which showed that ethiofos was readily hydrolyzed at a pH of 1.0). If such is the case, then it would be appropriate to protect ethiofos with an enteric coating for passage through the stomach. One convenient method of applying such a coating is microencapsulation, a process which may subject the drug to elevated temperatures. Therefore, prior to undertaking any microencapsulation studies, adequate thermal stability of ethiofos had to be established.

During the first year term of the contract, ethiofos was shown to be thermally stable and was successfully encapsulated as microspheres and as microcapsules. Several different matrices were established which protected the drug from acid hyrolysis yet would release it in solutions of pH 7.5. These formulations appeared promising as the initial candidates for oral dosing studies. However, prior to these studies a plasma assay for ethiofos had to be established and during the period covered by the third annual report an assay for ethiofos was developed such that animal dosing studies could proceed.

After the assays were completed for the initial dosing studies it became apparent that greater sensitivity in the detection of ethiofos was required and subsequently a more sensitive assay method was developed during the fourth year of the program.

In addition an assay method of WR 1065, the thiol metabolite of ethiofos, was needed and was developed for the unbound metabolite in plasma. An assay method for WR 33278, the disulfide metabolite of WR 1065, is also required and its development is in progress.

II. BACKGROUND

During the period covered by the first, second, third and fourth annual reports under this contract, investigations were conducted which demonstrated the following:

- the hydrolytic instability of ethiofos under acidic conditions but greater stability under alkaline conditions;
- the thermal stability of ethiofos when heated at 60°C under nitrogen for at least one hour;
- the successful encapsulation of ethiofos in a variety of glycerides, fatty acids and paraffins and mixtures thereof;
- the stability of ethiofos in certain encapsulated products in pH 1.0 solutions at 37°C for 1.5 hours;
- the release of ethiofos from certain of the promising encapsulated forms at pH 7.5 (37°C for 2 hours) using a buffered solution or synthetic intestinal fluid;
- the ability to directly assay microcapsules and some buffered solutions for ethiofos using an HPLC procedure developed at SwRI;
- the ability to analyze for ethiofos present in synthetic intestinal fluid using an alternate procedure which removes interferences in this system;
- the development of an improved plasma assay method for ethiofos over the range of 0.05 µg/mL to >1000 µg/mL with WR 80855 as an internal standard and successful application of the method in ethiofos dosing experiments using beagle dogs. Preliminary evaluation of the results indicated that the data were compatible with a recycling process with a clearance of about 0.3 L/hr/kg and an apparent terminal half-life of about 1.5 days.
- the development of an HPLC plasma assay method for WR 1065 over the range 1 µg/mL to >500 µg/mL.
- the application of the WR 1065 assay in preliminary dosings of ethiofos. Low concentrations of the metabolite were measured up to 720 minutes post infusion.

- the instability of WR 1065 in dog plasma even when samples are stored at 75°C. Methods of stabilizing the samples appeared promising in early investigations.

- the reproducibility of the encapsulation of ethiofos to give good protection of the drug against acid hydrolysis and rapid release at pH 7.5.

These results continue to justify the approach taken; that is, the development of an acceptable oral dosage form by encapsulation of the drug with enteric-type coatings which protect it from acid hydrolysis during passage through the stomach and then release the drug in the intestinal tract.

In order to develop and evaluate such a dosage form, the reported studies were undertaken.

III. EXPERIMENTAL

A. Analytical Methods Development

1. Apparatus

a. HPLC Unit A (WR 1065 assay). An IBM Model LC 9533 HPLC equipped with a Rheodyne 7125 injector was fitted with a Regis Reversible C8 5u analytical column, IBM Model EC/230 amperometric detector, Bioanalytical Systems mercury/gold transducer cell and an Omniscribe strip chart recorder to form the HPLC system.

b. HPLC Unit B (WR 33278 assay). An LDC Constametric III HPLC pump equipped with a Rheodyne 7125 injector was fitted with a Regis Reversible C8 5u analytical column, flow through pulse damper, two Bioanalytical Systems LC 4B amperometric detectors modified for dual electrode analysis and a dual mercury/gold transducer cell, and an Omniscribe strip chart recorder to form the HPLC system.

c. HPLC Unit C (WR 33278 assay). A Rainin Rabbit (TM) Model HP HPLC pump equipped with a Rheodyne 7125 injector was fitted with a Regis Reversible C8 5u analytical column, Rainin electronic pressure monitor, flow-through pulse damper, two Bioanalytical Systems LC 4B amperometric detectors modified for dual electrode analysis and a dual mercury/gold transducer cell connected to a Kipp and Zonen BD40 strip chart recorder to form the HPLC system.

d. HPLC Unit D (ethiofos assay). An LDC Constametric III Pump was fitted with a Rheodyne 7125 injector, and LDC Fluoromonitor III fluorescence detector, an LDC CI-10 integrator/recorder and a Waters and Associates RCM-100 radial compression module containing a 5µ C-18 reverse phase cartridge.

e. HPLC Unit E (ethiofos assay). A Waters and Associates model 6000A pump was connected to a Rheodyne 7125 injector, an LDC Fluoromonitor III fluorescence detector, a Waters Associates Data Module integrator/recorder and a Waters Associates RCM-100 radial compression module containing a 5µ C-18 reverse phase cartridge.

2. Solvents

Organic solvents were Fisher Brand or Baker Analyzed HPLC reagent grade. Water was purified with a Milliport Milli RO4 purification system.

3. Reagents

All reagents were ACS reagent grade. Dodecyltriethylammonium phosphate was purchased from Regis Chemical Company.

B. In Vivo Studies

The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards set forth by the "Guide for the Care and Use of Laboratory Animals" DHEW Publication, HIH74-23.

Healthy, male beagle dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, Michigan and healthy male rhesus monkeys were purchased from Argon Regional Primate Research Center, Beaverton, Oregon, for use in pilot dosing studies to test the analytical methods.

C. Microencapsulation Studies

1. Materials

The active materials being investigated in the encapsulation studies are:

Ethiofos Lot Nos. AX BK 02762 PB-V-116
AY BK 45589 AP-10-205
WR 2823 Lot No. AEAN 25574

The glycerides, fatty acids, oleic acid, soybean oil, sorbitol, and gelatin used for the shell coatings and fill carriers were standard food grade.

2. Procedures

The centrifugal extrusion process described in the Second Annual Report for the project was used to prepare the microspheres and microcapsules.

3. Evaluation

The hydrolytic stability and release rate studies of the microspheres and microcapsules were conducted according to the methods described in the Second Annual Report.

IV. RESULTS AND DISCUSSION

A. Analytical Methods Development

1. Ethiofos (WR 2721) Assay Modification

During this report period a paper entitled "An Improved HPLC Assay for S-2-(3-Aminopropylamino)ethyl Phosphorothioate (WR 2721) in Plasma" was published in the International Journal of Radiation Oncology, Biology, Physics, Vol. 10, pp 1521-1524 (1984). Authors were N.F. Swynnerton, E.P. McGovern, J.A. Niño and D.J. Mangold. A manuscript was submitted to the Journal of Pharmaceutical Sciences bearing the title "Measurement of Ethiofos (WR 2721) in Plasma: Preliminary Pharmacokinetics in the Beagle Dog". Authors were N.F. Swynnerton, D.J. Mangold and T.M. Ludden. The manuscript was drawn from Study Report 5 which detailed a modification of the ethiofos plasma assay to give greater selectivity for unchanged drug. Pharmacokinetic modeling was carried out using plasma concentration data obtained using the modified analytical procedure. The report is attached as Appendix A.

The modification to the method consisted of a change to the HPLC mobile phase system to separate derivatized drug and internal standard from an endogenous plasma component which interfered. The interference was small and usually corresponded to much less than 5 µg ethiofos per mL of plasma. The interference was not detected during development of the assay in which plasma from a variety of dogs and several beagles was analyzed. Only after IV administration of the drug did the interference become apparent, the result of which was the conclusion that a long terminal phase existed. The modified HPLC system has apparently separated the interference because repeat dosing experiments do not show a long terminal phase and reanalysis of earlier samples show lower (or no) ethiofos concentrations at extended postinfusion times.

2. Further Refinement of the HPLC Method for Ethiofos

Although the present procedure for the analysis of ethiofos in plasma has proved to be reliable and sensitive, several areas have been identified for improvement. With the expected sample load being large, an analysis time of 1 hour is not ideal. Additionally, the current ion pairing reagent, dodecyltriethylammonium phosphate (Q-12), may cause the earlier eluting endogenous materials to be incompletely separated from ethiofos and the internal standard.

This effect is manifested by a long tail on which are superimposed the peaks of interest (Figure 1). This posed no problem when higher levels of drug were present but became a problem when maximum sensitivity was required because the baseline was often offscale at time of elution of drug and standard. Decreasing the "strength" of the mobile phase to increase separation added to the total analysis time. Several approaches to solution of the problems have been taken--all have met with some success but none was totally satisfactory. The approaches were as follows:

(a) insertion of a switching valve to reverse mobile phase flow in column,

(b) use of a precolumn sample treatment to "clean up" the sample,

(c) use of an extraction technique to "clean up" the sample,

(d) precipitation with organic solvent prior to derivatization.

A six-way switching valve was inserted between the injector and the detector. The valve was actuated immediately after detection of ethiofos (which elutes after WR 80855 in this system) in order to backflush the system (Figure 2). It was thought that this procedure would remove all later-eluting components of the complex sample mixture in a length of time similar to the elution time of ethiofos. In this way no additional flush with a "stronger" mobile phase would be necessary thereby eliminating the need for an equilibration period as well as doing away with having to force all components of the sample mixture completely through the analytical column in order to clean out the system. In practice the backflush required roughly twice as long to cleanse the system as the time required to elute the peaks of interest. This translates to a run time for a single analysis of about 1.5 hours which is unacceptable.

The backflush system was modified to backflush only the analytical column while maintaining forward flow in the guard column. Equilibration times were excessively long--about one hour--when the mobile phase was used as the flush solvent. Investigations on the backflush techniques have been suspended.

Next, attempts were made to use a precolumn to remove endogenous materials. Plasma containing known amounts of drug and standard

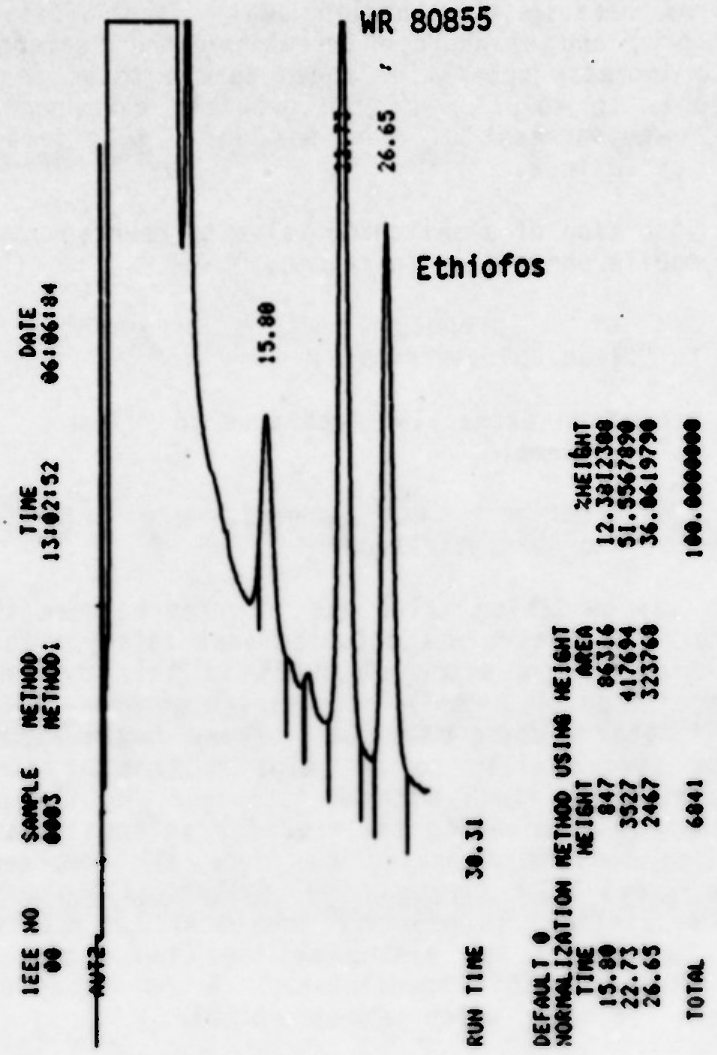
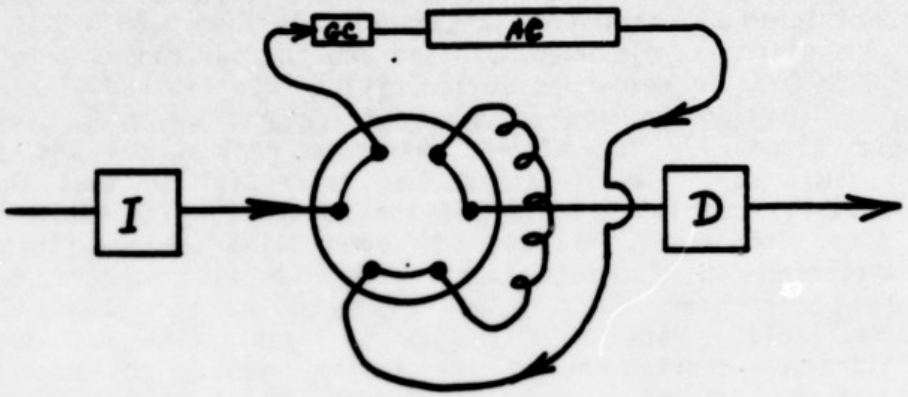
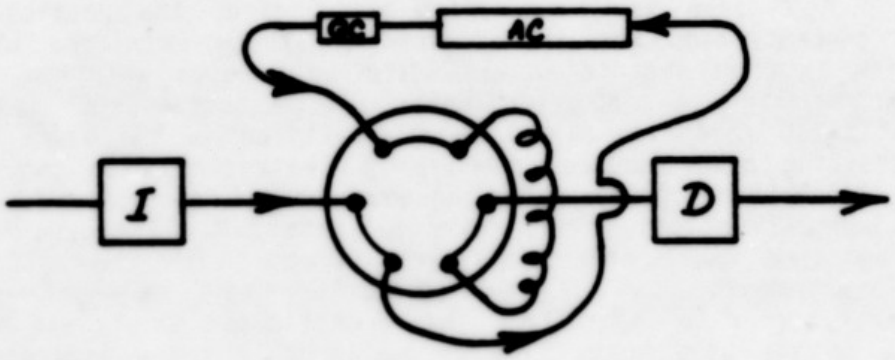


Figure 1. Beagle Plasma Spiked with 5 µg/mL Ethiofos and 5 µg/mL WR 80855.
Attenuation = 50×2^3

Position 1 - Normal operation



Position 2 - Backflush



I - injector
AC - analytical column
GC - guard column
D - detector

Figure 2. HPLC System with Six-Way Switching Valve for Backflush Operation

were treated with ion pairing agent [Q-12 (or Q-8, octyltriethylammonium phosphate)] and passed through cartridges containing C-18 reverse phase packing. Both the Waters and Associates Sep Pak™ and cartridges from J.T. Baker, Inc. were tested. The cartridges were conditioned by passing first acetonitrile then 0.005M Q-12 through them. The plasma samples were applied and the cartridges were flushed with 0.005M Q-12 to remove polar impurities then flushed with mixtures containing varying amounts of acetonitrile and water to remove the drug and standard. The eluant was then derivatized and analyzed in the usual way. The treatment was successful in that there was an essentially complete removal of the material which would normally elute after ethiofos. This was confirmed using the backflush system and monitoring the column eluant with the fluorescence detector. However, the recovery of drug and standard was less than 40% which was undesirable. Part of the loss of sensitivity was attributed to an inherent shortcoming of the system, namely the necessity to use at least 1 mL of solvent (preferably more) to remove the drug and standard from the cartridge. This results in a dilution of the sample with a consequential loss in sensitivity. This approach would be more applicable to larger and/or more concentrated samples, but appears to be of no use in our system, given its constraints.

A cleanup technique which would offer the possibility of sample concentration was investigated. Although ethiofos will not partition into an organic solvent from an aqueous solution, it was thought that ion pairing techniques might allow such a separation. To test this, fortified plasma was derivatized in the usual way and the resulting clear neutral mixture was extracted with two volumes of dichloromethane (DCM). The aqueous phase was removed, treated with 0.05M Q-12 solution, and extracted with DCM. The organic layer was evaporated under argon and reconstituted with the HPLC mobile phase in an amount equal to the volume of a normal, derivatized sample immediately prior to injection. The reconstituted sample was analyzed by HPLC in the usual way. The chromatographic trace indicated that the sample was much cleaner and that the "tailing" previously observed was no longer present. Recovery was about 25% of each component, with peak height ratios being the same as was observed in a control sample which was analyzed in the usual way (Figures 3a and 3b). When the experiment was repeated on a sample containing 1.0 µg ethiofos/mL of plasma the recovery was about 10%. The low recoveries were thought to result, at least in part, from the lengthy procedures in which the labile drug was in solution and in the presence of reactive materials at room temperature.

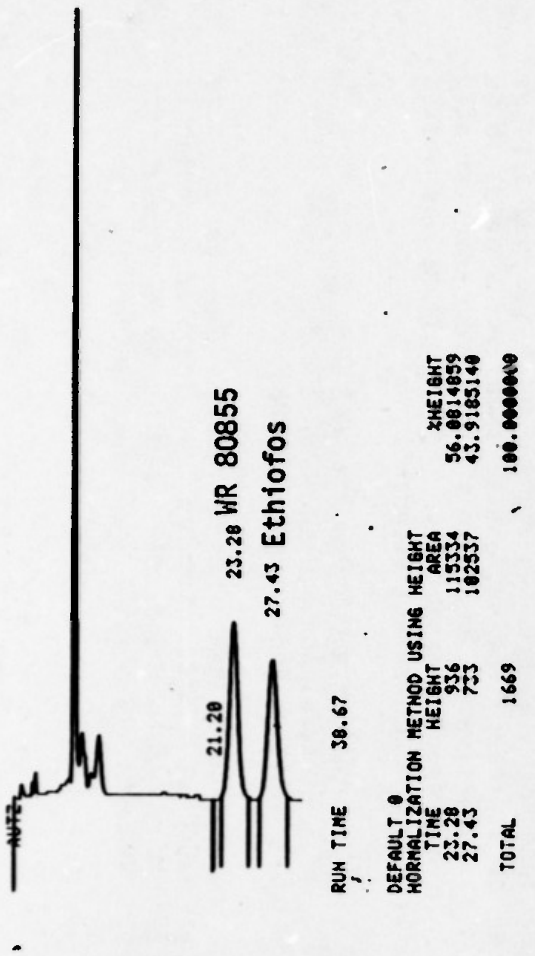


Figure 3a. Chromatographic Trace of Beagle Plasma Spiked with 50 µg/mL Ethiofos and 80 µg/mL WR 80855. Sample cleanup by DCM extraction. Attenuation = 5 x 100 x 10^3

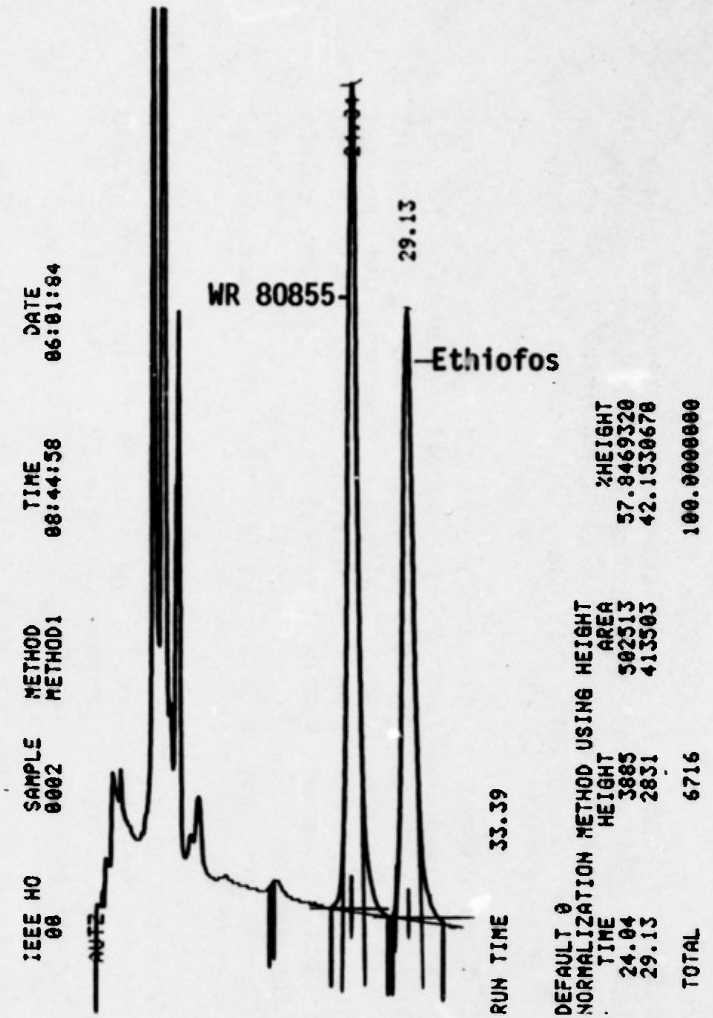


Figure 3b. Chromatographic Trace of Beagle Plasma Spiked with 50 µg/mL Ethiofos and 80 µg/mL WR 80855. Analysis by standard procedure. Attenuation = 5 x 100 x 10^3

Addition of acetonitrile to plasma samples prior to derivatization has resulted in cleaner samples as shown by the absence in chromatograms of the "tail" of earlier eluting material. The cleanup has also resulted in diminished response of the drug which reduced the minimum detectable limit to about 0.2 µg/mL.

The disadvantages of the procedures to further refine the ethiofos HPLC assay have not been overcome, partly due to a reduced effort necessitated by a shift of emphasis to animal dosing experiments and the need to carry out plasma analyses. However, our present chromatographic separation of drug from internal standard is excellent (R_S=2.3) and total time of sample preparation, elution and column re-equilibration is about one hour. Precision and accuracy are good and the pharmacokinetic and bioavailability studies can be carried out with the present system.

During this period another method for analysis of ethiofos in plasma appeared in the literature.[1] The authors claim a more rapid analysis time with slightly poorer sensitivity. The method will be evaluated in our laboratories.

3. Stability of Ethiofos and WR 80855 in Stored Beagle Plasma

We previously reported (13th Quarterly Report and Chemical Modifiers of Cancer Treatment Conference, Banff, Alberta, Canada, November 17-December 1, 1983) that ethiofos present in beagle plasma decomposed during storage at -20°C. It was also reported that the rate of decomposition was dependent upon the amount originally present, lower concentrations suffering the greatest decomposition. Further, we reported that samples suffered no measurable decomposition after 18 weeks of storage at -75°C. These results were relative to the stability of the internal standard, WR 80855, which was added prior to the beginning of storage. The "absolute" stabilities of ethiofos and WR 80855, i.e., relative to an external standard, are currently being estimated in an on-going experiment. Beagle plasma was spiked at three levels with drug and internal standard:

Level 1 0.5 µg/mL ethiofos and 0.8 µg/mL WR 80855
Level 2 5.0 µg/mL ethiofos and 8.0 µg/mL WR 80855
Level 3 50 µg/mL ethiofos and 80 µg/mL WR 80855

After dividing the spiked plasma into aliquots, the samples were stored at either -20°C or -75°C. Samples were analyzed immediately for baseline (zero time) values and other samples were removed from storage at 3 weeks, 8 weeks, 18 weeks and 26 weeks immediately analyzed

in triplicate for ethiofos and WR 80855. Repeatability of the analyses was excellent with an average coefficient of variation of 38% for 21 sets of replicates.

(a) Stability of Ethiofos Relative to WR 80855

The relative stabilities of the drug and internal standard were estimated by comparing the ratio of their peak heights (HPLC chromatographic analysis) at the time intervals. Results were in good agreement with those obtained previously, i.e., samples stored at -75°C gave ratios unchanged by storage, while those stored at -20°C suffered a decrease in ethiofos relative to WR 80855. Again, samples with the lower original levels of ethiofos showed greater losses of drug relative to the standard. Plots of the data collected through 26 weeks are presented in Figures 4 and 5.

(b) Absolute Stability of Ethiofos

The "absolute stability" of ethiofos stored in frozen plasma at -20°C and -75°C was estimated by comparing the assay of the stored samples with a freshly prepared plasma sample spiked to the same levels. Values for ethiofos concentrations at the various time intervals are presented in Table 1. Precision and accuracy were poorer than with the relative measurements for obvious reasons. Even considering the scatter in the data, two trends are evident: (1) lower levels of ethiofos degrade more rapidly and (2) samples stored at -75°C are more stable. The values for the samples stored at -75°C for 26 weeks, although slightly lower than the time zero values, may be the same within experimental error. The test will be continued through 12 months.

4. Free WR 1065

a. Method Development

The plasma assay method for WR 1065 using liquid chromatography with electrochemical detection (LCEC)[2] has been modified to increase sensitivity. The method is precise and accurate (Tables 2 and 3) and allows for detection of less than 0.05 µg/mL of WR 1065 in plasma. Details of the method and its application to pharmacokinetics are presented in Study Report 6, attached as Appendix B.

Longer column life has resulted from improved protein precipitation during sample preparation. Pretreatment of samples with a mixture of 0.05 M 5-sulfosalicyclic acid and mobile phase

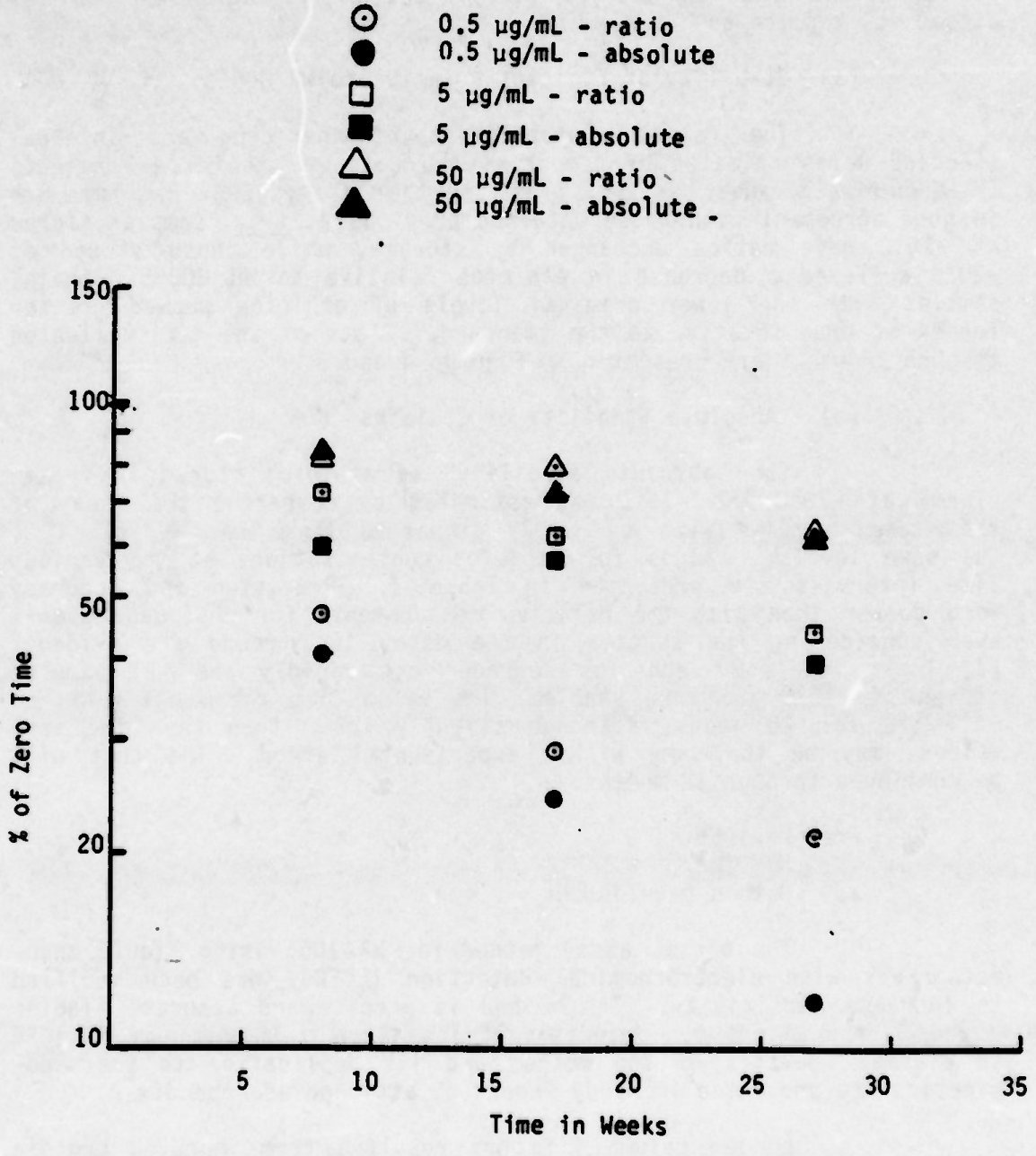


Figure 4. Stability of Ethiofos in Plasma at -20°C.

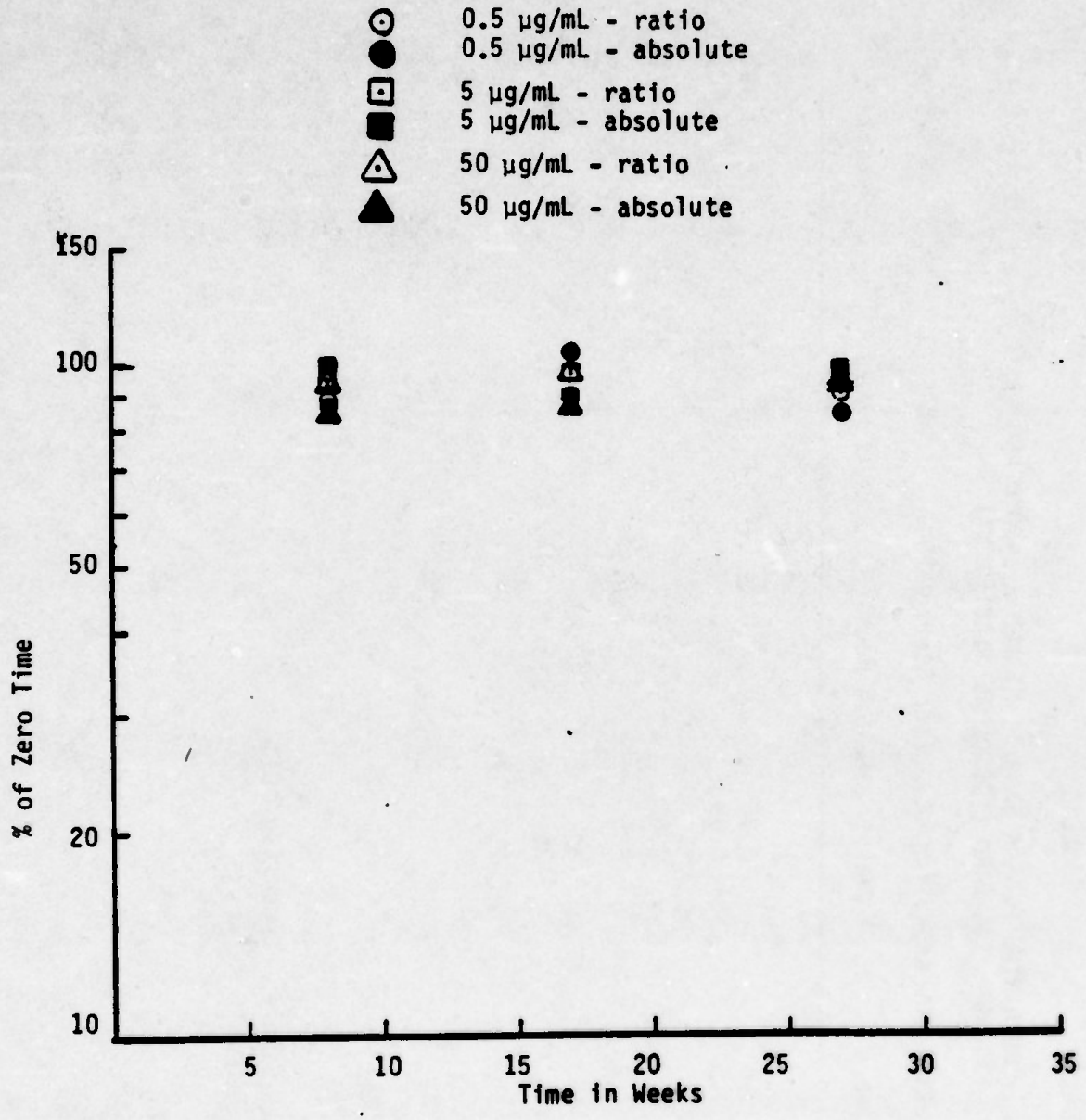


Figure 5. Stability of Ethiofos in Plasma at -75°C.

TABLE 1

ASSAY OF ETHIOFOS-CONTAINING PLASMA SAMPLES STORED AT -20°C AND -75°C USING EXTERNAL STANDARD ("ABSOLUTE STABILITY")

Temperature, °C	Ethiofos Assay (µg/mL) After Storage				
	Zero Time	4 Weeks	8 Weeks	17 Weeks	27 Weeks
-20	0.5	0.36	0.21	0.12	0.06
-75	0.5	0.69	0.44	0.58	0.42
-20	5.0	4.8	3.0	2.9	2.0
-75	5.0	4.2	5.0	4.5	4.8
-20	50	42	42	37	31
-75	50	36	43	44	48

Calculation: $\frac{\text{Conc. of Std. (µg/mL)}}{\text{Pk. Ht. of Std.}} = R_F$

R_F x Pk Ht of Test Sample = Conc. (µg/mL)

TABLE 2

PRECISION ESTIMATES FOR WR 1065 ASSAY USING WR 1729 AS AN INTERNAL STANDARD

	Plasma Level of WR 1065 µg/mL					
	0.05	0.1	0.5	1.0	5.0	50.0
Number of replicates	5	5	5	4	5	5
Standard deviation	0.11	0.02	0.01	0.06	0.46	0.39
Coefficient of variation, %	11.1	7.5	1.3	3.9	6.3	10.6
Internal standard concentration, µg/mL	1	1	1	1	1	10

TABLE 3

ACCURACY OF WR 1065 ANALYTICAL METHOD

Spike Level µg/mL	Measured Level µg/mL	Percent Deviation (D)
0.1	0.09	-7.0
0.2	0.21	+5.0
0.7	0.65	-7.1
0.9	0.88	-2.2
2.0	1.95	-2.5
5.0	4.83	-3.4
25.0	25.6	+2.4
35.0	33.4	-4.6
50.0	51.6	+3.2

$$\text{Average Deviation} = \frac{\Sigma_1^n |D|}{n} = 4.2$$

reduces endogenous material which otherwise would have been injected into the column. This eliminates the need to "flush" the column after each sample injection resulting in shorter analysis times of about 20 minutes.

5. Plasma Covalently Bound WR 1065

Recent studies were directed toward development of an analytical method to quantitate WR 1065 bound to plasma components through disulfide bonds. An assay method has been developed which uses tributylphosphine to reductively cleave the disulfide bonds. An apparent five-fold increase in WR 1065 concentration (relative to an untreated sample) was observed after a plasma sample (60 minutes post ethiofos infusion) was treated with a methanolic tributylphosphine solution prior to analysis (Figure 6). Further studies using this method are planned which will provide more information regarding covalently bound WR 1065.

6. WR 1065 Stability in Plasma

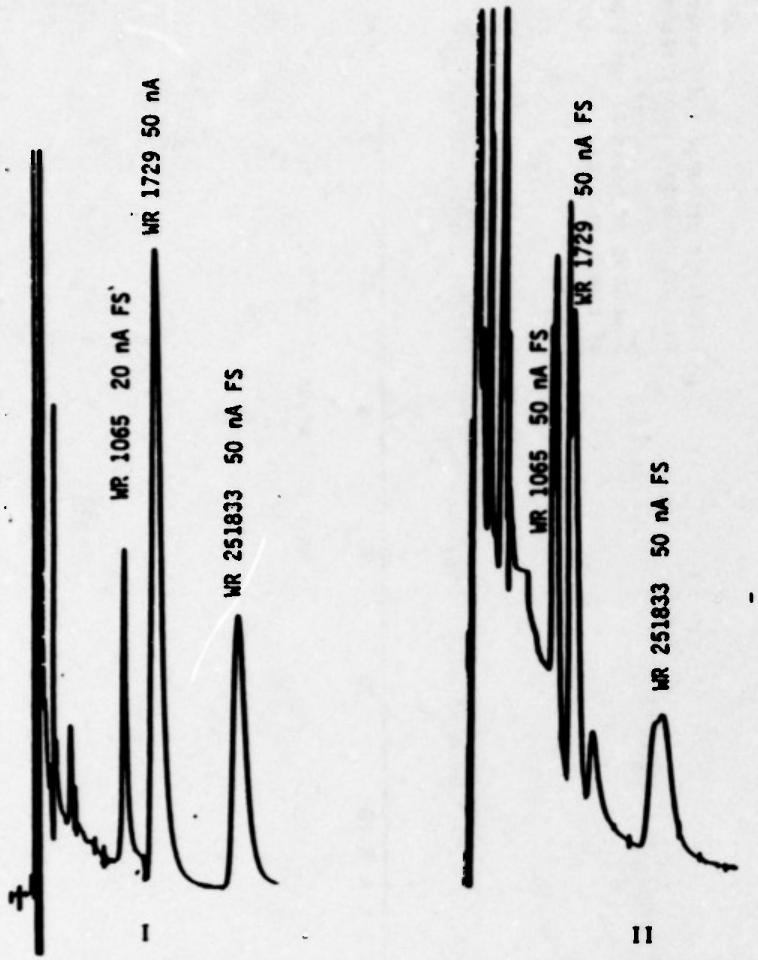
Figure 7 illustrates the results of the analysis of preserved plasma samples fortified at the time of preparation with 100 µg/mL of WR 1065 and WR 1729. Both compounds exhibit the same relative stabilities during storage at -75°C for 55 days.

7. WR 33278

a. Method Development

An HPLC plasma assay has been developed for the disulfide WR 33278. The assay employs a disulfide specific mercury/gold dual electrode electrochemical detector. The electrochemical cell is configured with the electrodes in series. upon traversing the upstream electrode, WR 33278 is reduced to its thiol form. The thiol is then detected at the downstream electrode (Figure 8 is a drawing of the cell used in HPLC Units B and C).

Separations were carried out using a Regis C8 Reversible 5u analytical column. The mobile phase was acetonitrile/0.1M monochloroacetic acid (11:89), and 0.1M camphorsulfonic acid (pH 3) at a flow rate of 1.0 mL/minute. Various disulfide compounds were tested for use as an internal standard but the disulfide of WR 1729, WR 149024, was found to be the most suitable for this application. WR 33278 and WR 149024 elute at 12.0 and 16.4 minutes, respectively with this chromatographic system (Figure 9). The peak height ratio



Injection of
50 µL of 60 minute post
WR 2721 infusion sample

1. Before treatment with tributylphosphine
2. After addition of 100 µL of a 2% methanolic tributyl phosphine solution

Figure 6. Increase in WR 1065 Concentration Upon Sample Treatment with Tributylphosphine.

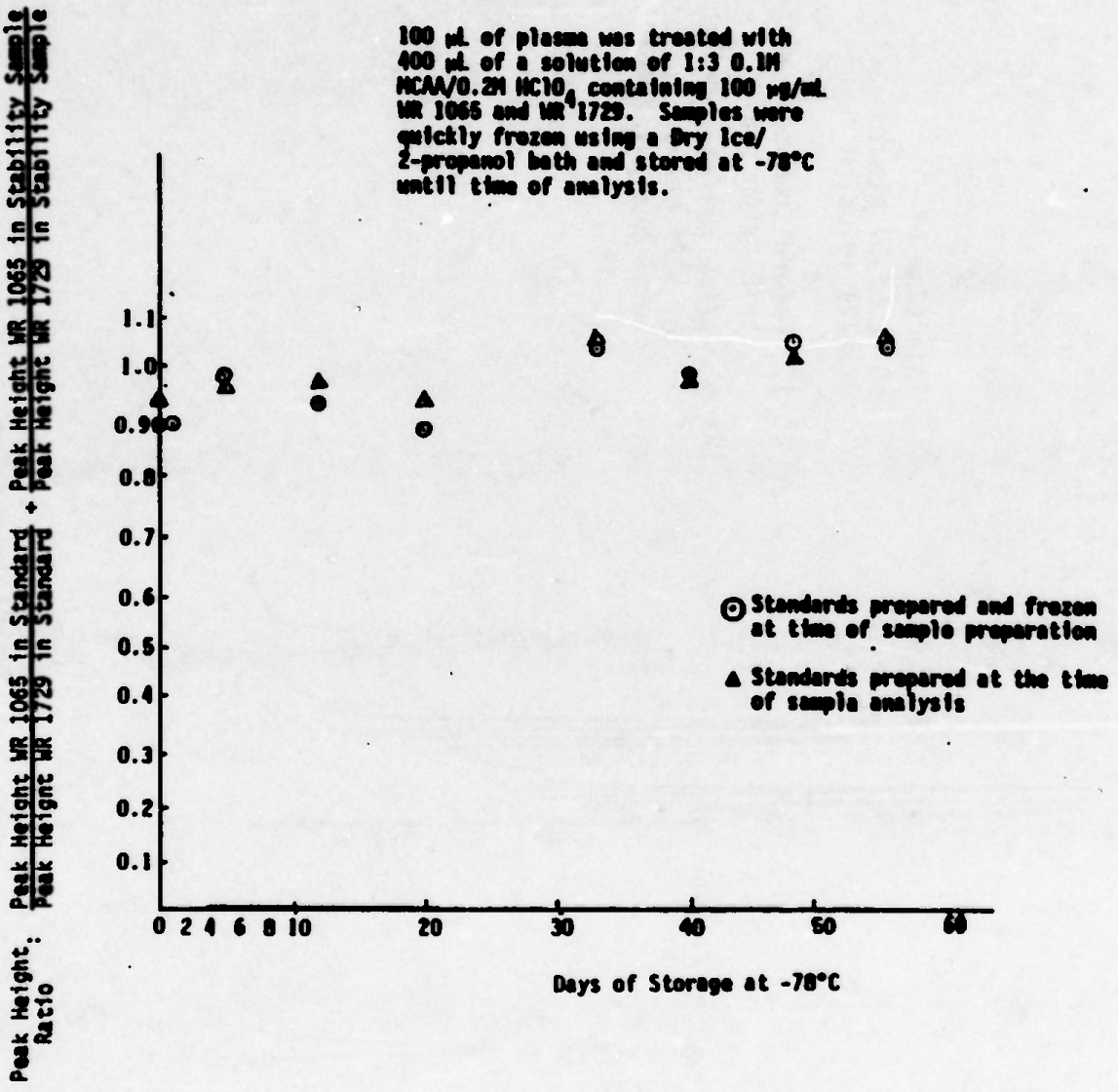
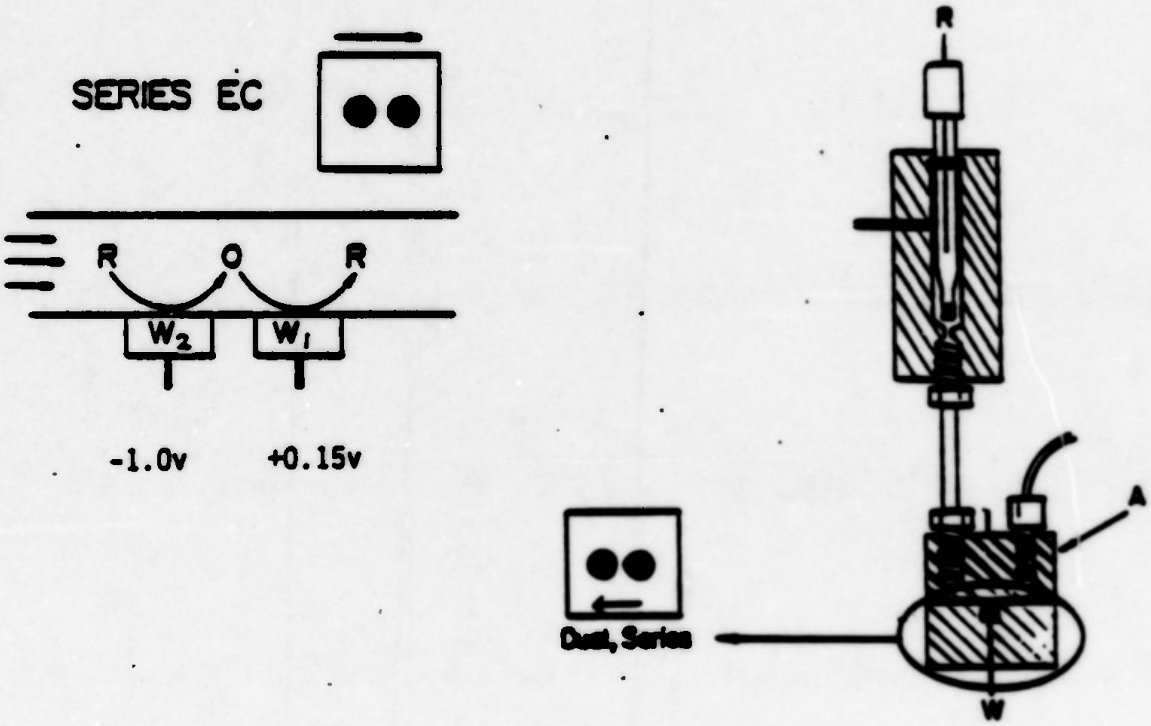


Figure 7. Stability of WR 1065 and WR 1729 in Beagle Plasma Treated with 0.1M Monochloroacetic Acid/0.2 Perchloric Acid 1:3 and Stored at -78°C.

$$RSSR + 2H^+ + 2e^- \longrightarrow 2RSH \qquad -1.0v$$

$$2RSH + Hg^0 \longrightarrow HgSR + e^- + H^+ \qquad +.15v$$

$$2HgSR \longrightarrow Hg^0 + Hg(SR)_2$$



R - Reference electrode
A - Auxillary electrode
W - Hg/Au working electrode

Figure 8. Electrochemical cell configuration for the detection of disulfides.

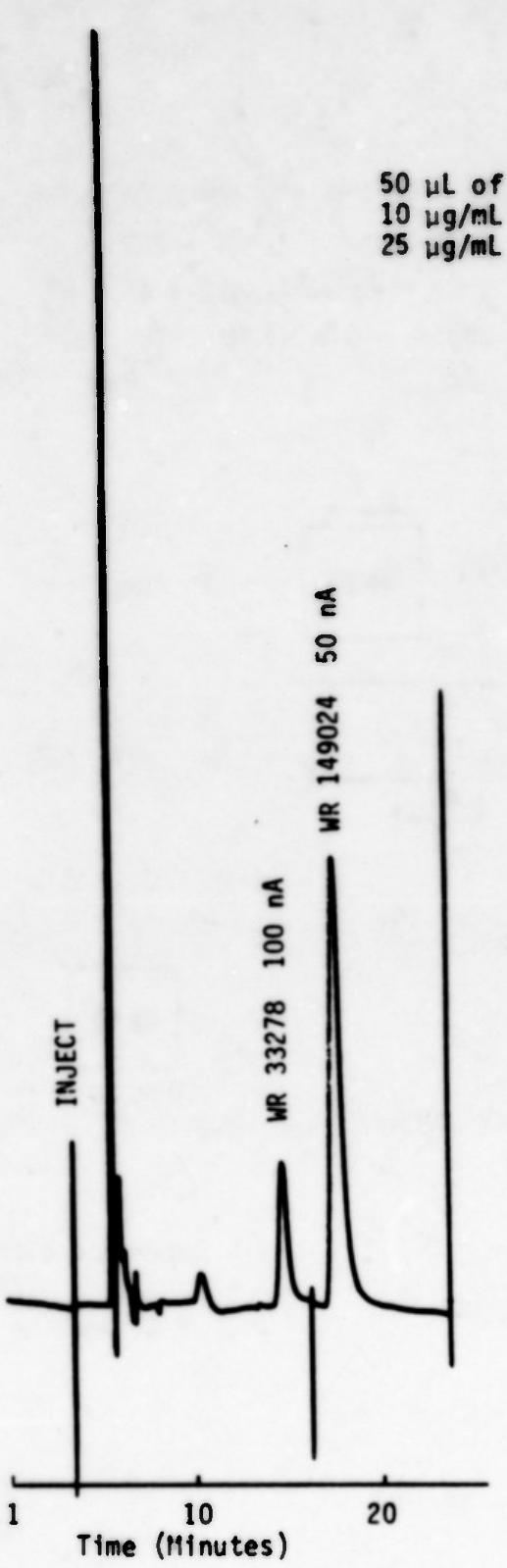


Figure 9. WR 33278 Plasma Spike

versus concentration calibration curve was found to be linear over the WR 33278 concentration range 0.5 to 100 µg/mL (Figure 10).

Precision and accuracy determinations were conducted at various concentration levels of WR 33278 (Tables 4 and 5). The blind accuracy samples were prepared by L. Fleckenstein during site visit on 11/29/84 (Table 6). The precision and accuracy data determined by SwRI personnel are acceptable; however, there exists a positive bias in the results from analyses of samples prepared by L. Fleckenstein. This bias suggests an interlaboratory procedural difference in sample preparation.

b. Relative Stability in Plasma

WR 33278 (5 µg/mL) in plasma was found to be relatively stable (2.68 RSD) for at least 2 hours at 4°C (Table 7; Figure 11). A long term stability study of 10 µg/mL WR 33278 in plasma, stored at -75°C, is now in progress. After 24 days, a 13.5% difference between peak height ratios of drug to internal standard was obtained for stored samples and daily standards.

c. Preliminary Dog Dosing Studies

Two preliminary IV WR 33278 dog dosings have been conducted to date. The drug concentration versus time profile for dog dosing no. 40 is presented in Figure 12 and samples from dosing no. 39 are currently being analyzed. Plasma concentrations of WR 33278 decreased rapidly to less than 5 µg/mL within the first 20 minutes post infusion. Time periods beyond 40 minutes showed <0.1 µg/mL WR 33278 present in the plasma.

B. In Vivo Studies

A total of thirty-four dosing experiments were carried out during this reporting period. Ethiofos was administered in twenty-five experiments, WR 1065 in seven experiments and WR 33278 in two experiments. Four beagle dogs and four rhesus monkeys were used in the dosings. A summary of all dosings is presented in Table 8.

1. Ethiofos Dosings

Ethiofos was administered to beagle dogs in thirteen experiments, seven oral and six intravenous. Twelve administrations of the drug were to rhesus monkeys, six oral and six intravenous.

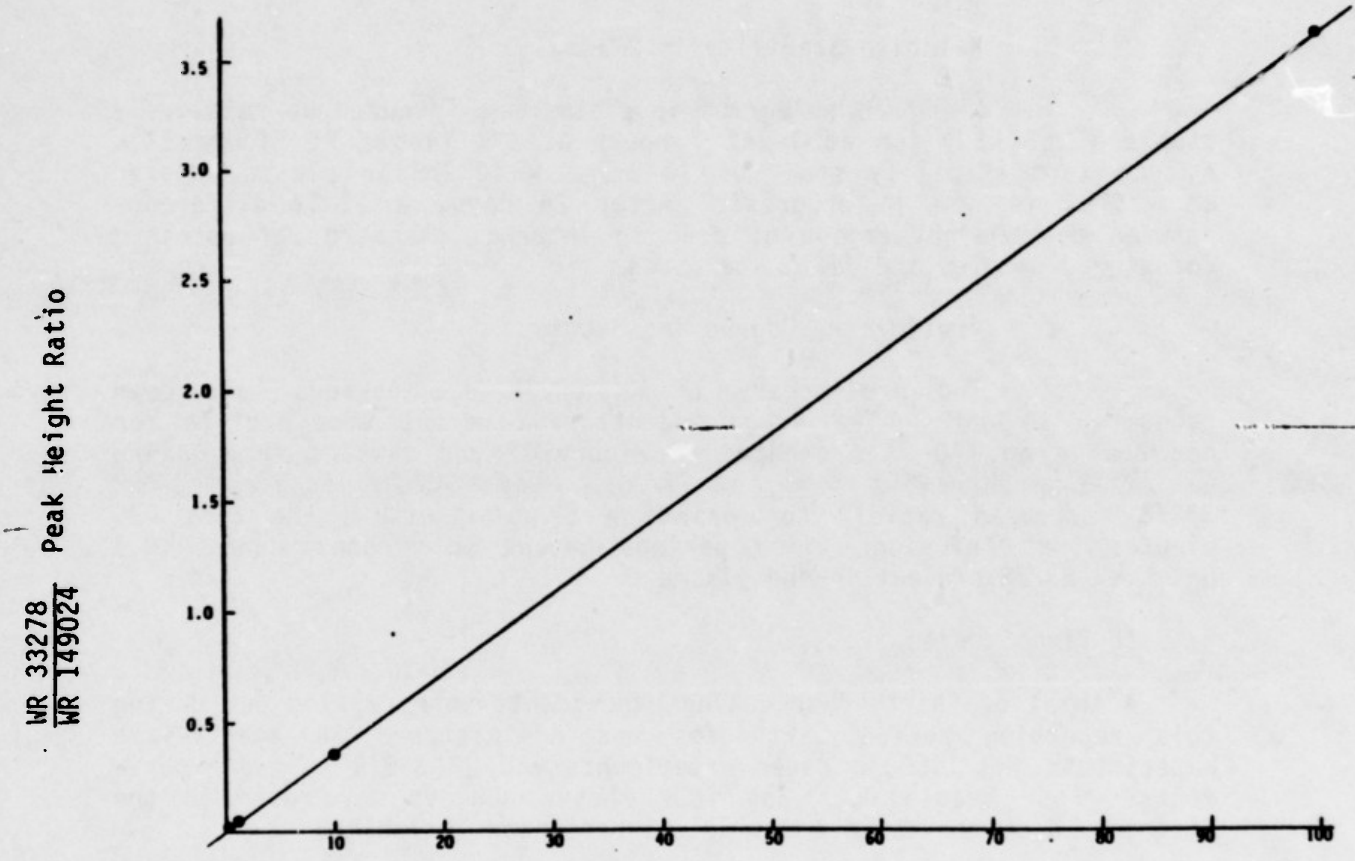


Figure 10. Calibration curve for WR 33278 0.5 to 100 ug/mL.

TABLE 4

PRECISION ESTIMATE FOR WR 33278 ANALYTICAL METHOD

Concentration µg/mL WR 33278	Average Ratio*	Standard Deviation	Relative Standard Deviation
1.0	0.10	0.01	4.90
5.0	0.27	0.02	6.26
10	0.53	0.01	1.95
25	1.41	0.10	7.38
50	2.62	0.13	5.03
100	5.33	0.11	2.03

*WR 33278 peak height/WR 149024 peak height

TABLE 5

ACCURACY ESTIMATE FOR WR 33278
DETERMINED IN HOUSE

Known Concentration WR 33278 µg/mL	Estimated Concentration µg/mL	% DEV
1.0	1.02	+2
	1.08	+8
	1.11	+11
5.0	5.64	+13
	5.34	+7
	4.94	-1
10	10.2	+2
	10.4	+4
	10.4	+4
25	22.7	+9
	26.8	+7
	26.4	+6
50	55.1	+10
	52.4	+5
	55.9	+12
100	102	+2
	104	+4
	99.4	+.6

TABLE 6

ACCURACY STUDY/BLIND SAMPLES PREPARED BY L. FLECKENSTEIN

Sample No.	Known Concentration WR 33278 µg/mL Plasma	Estimated Concentration WR 33278 µug/mL Plasma	% Deviation From Known Concentration
1	7.58	7.69	+1.5
2	75.8	107	+41.2
3	7.58	10.8	+42.5
4	75.8	112	+47.8
5	7.58	13.0	+71.5
6	75.8	108	+42.5
7	0.76	0.58	-23.7
8	Blank	<0.1	------
9	75.8	116	+53.0
10	7.58	8.92	+17.7
11	75.8	110	+45.1
12	7.58	3.26	-57.0
13	0.76	1.42	+86.8
14	0.76	1.16	+52.6
15	7.58	9.05	+19.4
16	0.76	0.89	+17.1
17	0.76	0.95	+19.0
18	75.8	110	+45.1
19	7.58	11.4	+50.4
Blank		0.1	

TABLE 7

STABILITY OF MR 33278 IN PLASMA AT 4°C

50 µg/mL MR 33278 and MR 149024

Time Minutes	Peak Height (mm) MR 33278	Peak Height (mm) MR 149024	Ratio* MR 33278/MR 149024
0	67	50	1.34
30	63	47	1.34
85	62	44	1.41
110	63	45	1.40
140	62	44	1.41

Average Ratio	1.40
Standard of Deviation	0.04
Relative Standard Deviation	2.86.

*Average Peak Height Ratios

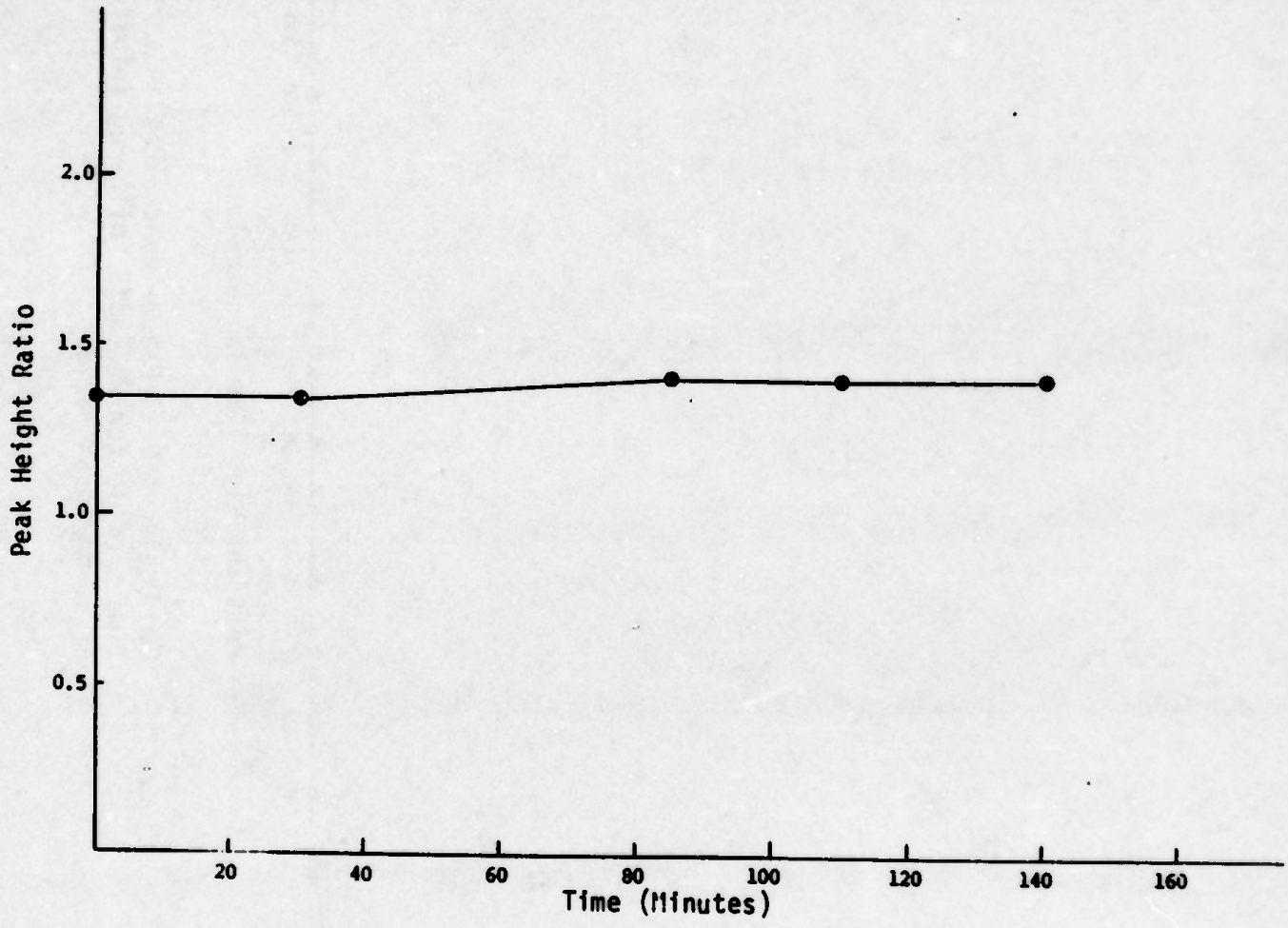


Figure 11. 50 ug/mL WR 33278 in Plasma 4°C

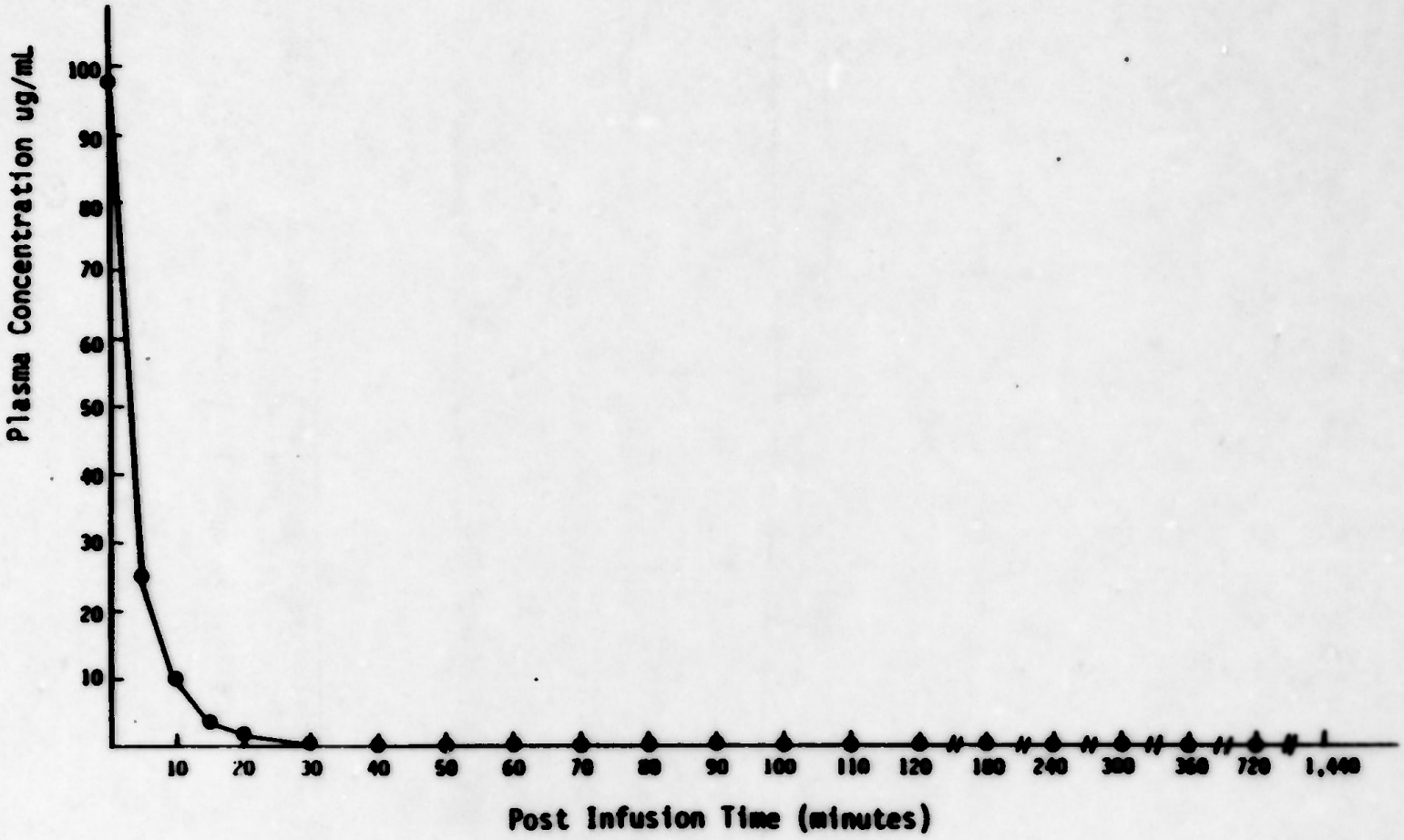


Figure 12. Dog Dosing #40 -- Plasma concentration versus time profile for WR 33278 following infusion of 60 mg/kg WR 33278.

TABLE 8

SUMMARY OF DOSINGS

Dosing No.	Dosing Date	Drug	Method of Administration	Dosing Level mg/kg[a]	Dosing Level µmol/kg[b]	Animal Registration No. (SwRI Identification)
9	1/11	WR 1065	IV	39	289	Beagle BO-13 (2)
10	1/25	ethiofos	"	150	70	Beagle CSX-1 (1)
11	2/8	"	Oral[c]	"	"	Beagle BO-13 (2)
12	2/29	"	"	75	350	Beagle CSX-1 (1)
13	3/7	"	"	150	700	Beagle BO-13 (2)
14	3/28	"	IV	"	"	Beagle ET-13 (3)
15	4/5	"	"	15,30[d]	70,140[d]	Beagle CSX-1 (1)
16	4/11	"	Oral	75	350	Beagle BD-13 (2)
17	5/2	"	IV	150	700	Beagle ET-13 (3)
18	5/9	"	"	"	"	Beagle CSX-1 (1)
19	5/16	"	Oral	"	"	Beagle BD-13 (2)
20	6/13	"	IV	"	"	Rh. Monkey 12072 (1)
21	6/20	"	Oral	"	"	Beagle ET-13 (3)
22	6/26	WR 1065	IV	39	289	Beagle CSX-1 (1)
23	6/27	ethiofos	"	200	934	Rh. Monkey 12012 (2)
24	7/11	"	Oral	"	"	Rh. Monkey 12072 (1)
25	7/12	WR 1065	IV	39	289	Beagle BO-13 (2)
26	7/18	ethiofos	Oral	150	700	Beagle ET-13 (3)
27	8/8	"	Oral[e]	"	"	Rh. Monkey 12012 (2)
28	8/16	"	IV	"	"	Beagle CSX-1 (1)
29	8/29	"	Oral	200	934	Rh. Monkey 12205 (3)
30	9/5	WR 1065	IV	39	289	Beagle ET-13 (3)
31	9/20	"	"	"	"	Beagle CSX-1 (1)
32	9/27	ethiofos	Oral	200	934	Rh. Monkey 12250 (4)
33	10/3	WR 1065	IV	39	289	Beagle BO-13 (2)
34	10/11	ethiofos	"	150	700	Rh. Monkey 12072 (1)
35	10/12	"	Oral	400	1867	Rh. Monkey 12012 (2)
36	10/16	WR 1065	IV	39	289	Beagle ET-13 (3)
37	10/17	ethiofos	"	150	700	Rh. Monkey 12205 (3)
38	10/24	"	"	"	"	Rh. Monkey 12250 (4)
39	10/31	WR 33278	"	64[f]	240[f]	Beagle CSX-1 (1)
40	11/7	"	"	16	60	Beagle BO-13 (2)
41	11/15	ethiofos	Oral	400	1867	Rh. Monkey 12072 (1)
42	12/13	"	IV	180	840	Rh. Monkey 12012 (2)

a. Weight of drug expressed on anhydrous, free base basis.
b. Concentration of drug expressed on anhydrous, free base basis.
c. All oral dosings except no. 27 were of the microencapsulated drug contained in two-part gelatin capsules.
d. Sequential doses given 180 minutes apart.
e. Neat ethiofos trihydrate contained in two-part gelatin capsules.
f. Planned dose. Actual delivery was about 16 mg/kg over a five-minute period. Infusion was stopped when animal suffered respiratory embarrassment.

Plasma ethiofos concentrations versus time for the oral dosings of beagles and rhesus monkeys are presented in Figures 13 and 14, respectively. In none of the experiments did the plasma concentration of ethiofos exceed 1.1 µg/mL, even with doses of 400 mg/kg administered. No drug was detected (lower limit of detection = 0.05 µg/mL) in six of the thirteen experiments.

Initial concentrations following intravenous administration of the drug (150-200 mg/kg over ten minutes) approached 800 µg/mL in both animals. The levels then dropped rapidly over one to two hours to near the lower limit of detection [Figures 15-18 (beagle) and 19-23 (rhesus monkey)]. When 150 mg/kg was infused over 110 minutes plasma concentrations near 100 µg/mL were measured during infusion. The concentrations fell rapidly postinfusion to the detectable limit of the analytical method within eight hours (Figure 24). Pharmacokinetic modeling of the data from the beagle IV experiments was carried out and reported in Study Report 5 (Appendix A).

2. WR 1065 Dosings

Seven WR 1065 intravenous dog dosing studies were conducted in 1984. The plasma drug concentration versus time profiles are presented in Figures 25-31. Three of the experiments (Dosing Studies 31, 33, 36) utilized the same methodology and the data obtained from them was used to prepare a pharmacokinetic model for this drug in the beagle (Table 9). All profiles show a rapid decrease in WR 1065 plasma concentration following an administration of 60 mg/kg. A plasma WR 1065 concentration versus time profile constructed from analysis of samples treated with tributylphosphine support the contention that the drug is quickly bound to plasma components (dog dosing no. 30, Figure 28).

Additionally, WR 1065 plasma levels have been measured following ethiofos dosing of both dog and monkey subjects. Dosings were ethiofos saline solutions given intravenously or encapsulated ethiofos given orally. Figure 32 presents results of beagle plasma analyses for WR 1065 following a 10 minute infusion of ethiofos. Maximum concentration levels (20-40 µg/mL) of WR 1065 occurred within the first 20 minutes post infusion, then rapidly decreased to 1 µg/mL or less for the final sampling periods. Figures 33 and 34 present results of rhesus monkey plasma analyses for WR 1065 following 10 minute infusions of ethiofos. Plasma concentration levels in the monkey also decrease rapidly within the first 20 minutes post infusion. Maximum concentration levels obtained were 20 to 60 µg/mL in plasma. Figures 35 and 36 present results of beagle plasma analysis for WR

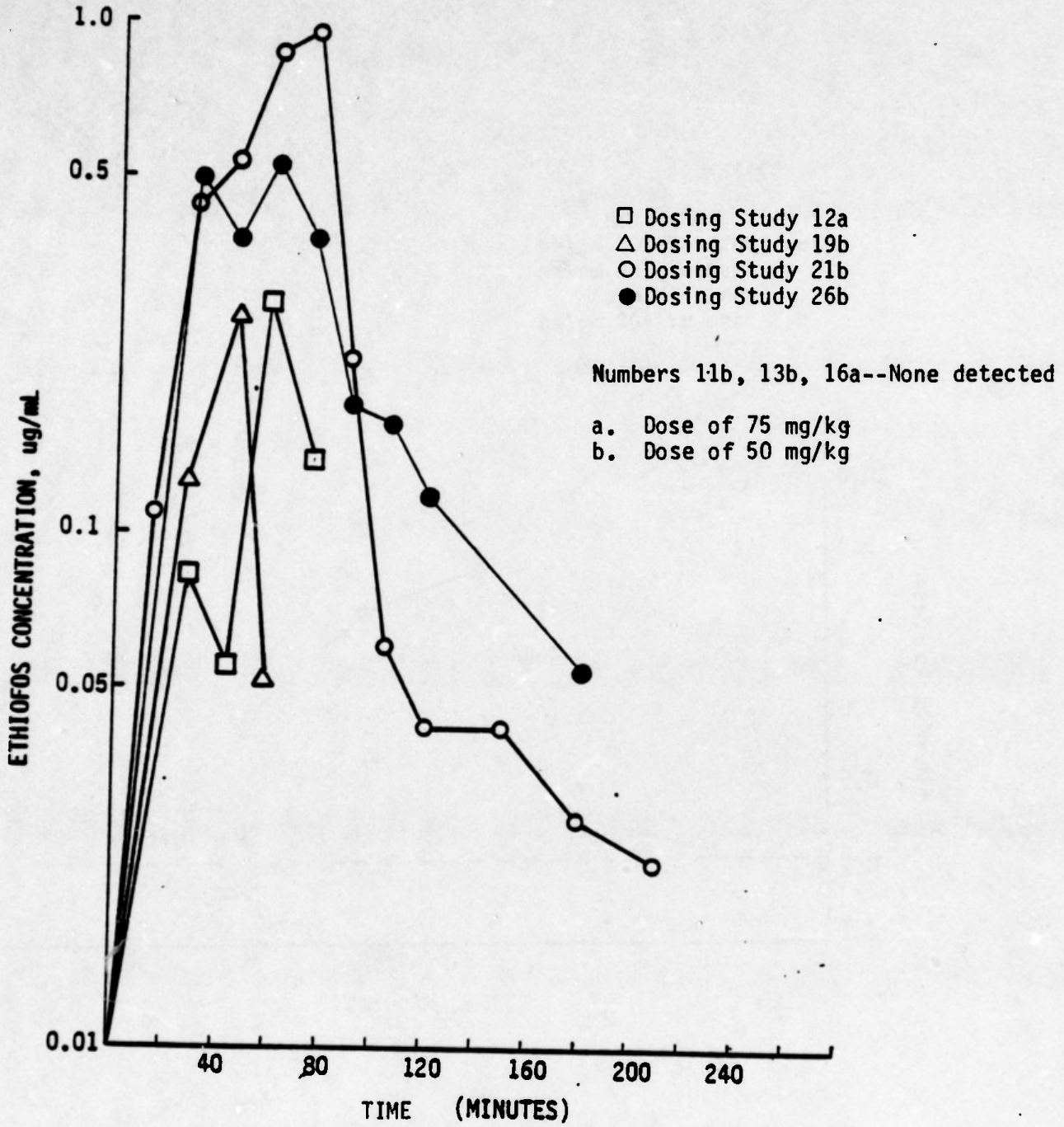


Figure 13. Plasma Ethiofos Levels Following Oral Dosings of Beagle Dogs

a. Dose of 200 mg/kg
b. Dose of 150 mg/kg
c. Neat Ethiofos
d. Dose of 400 mg/kg

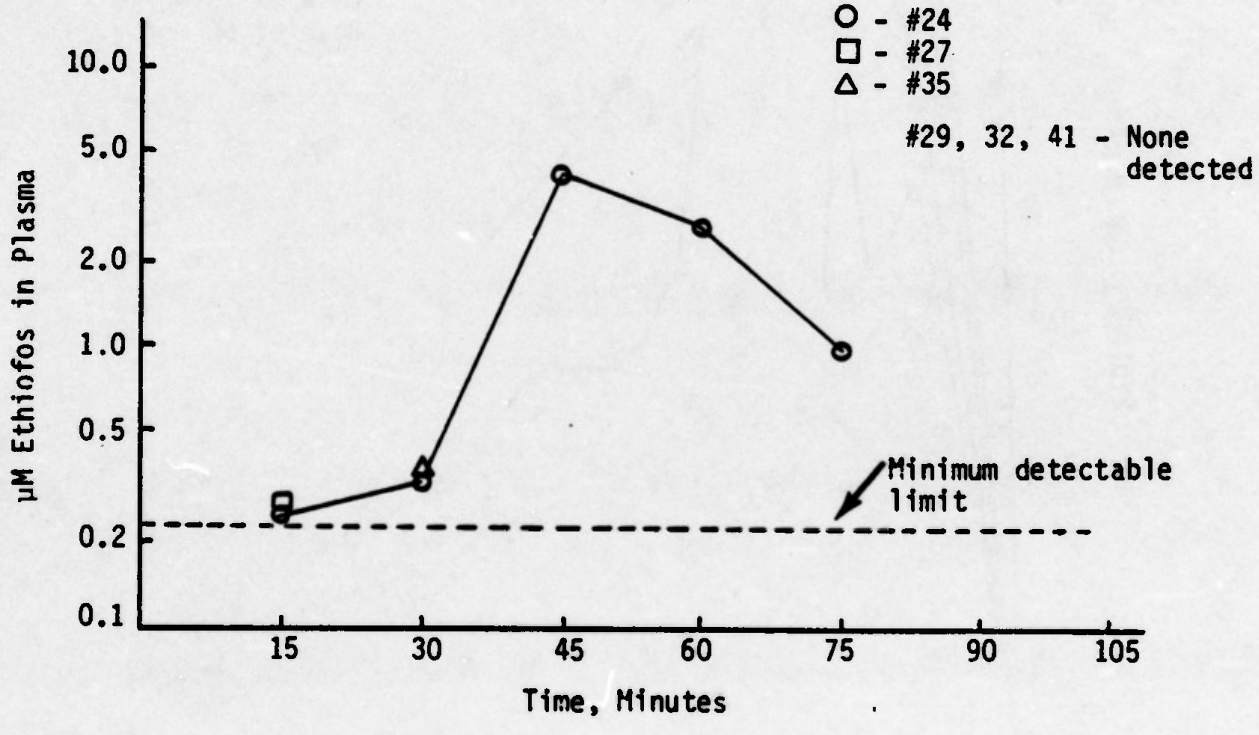


Figure 14. Plasma Ethiofos Concentrations Following Oral Dosings of Rhesus Monkeys.

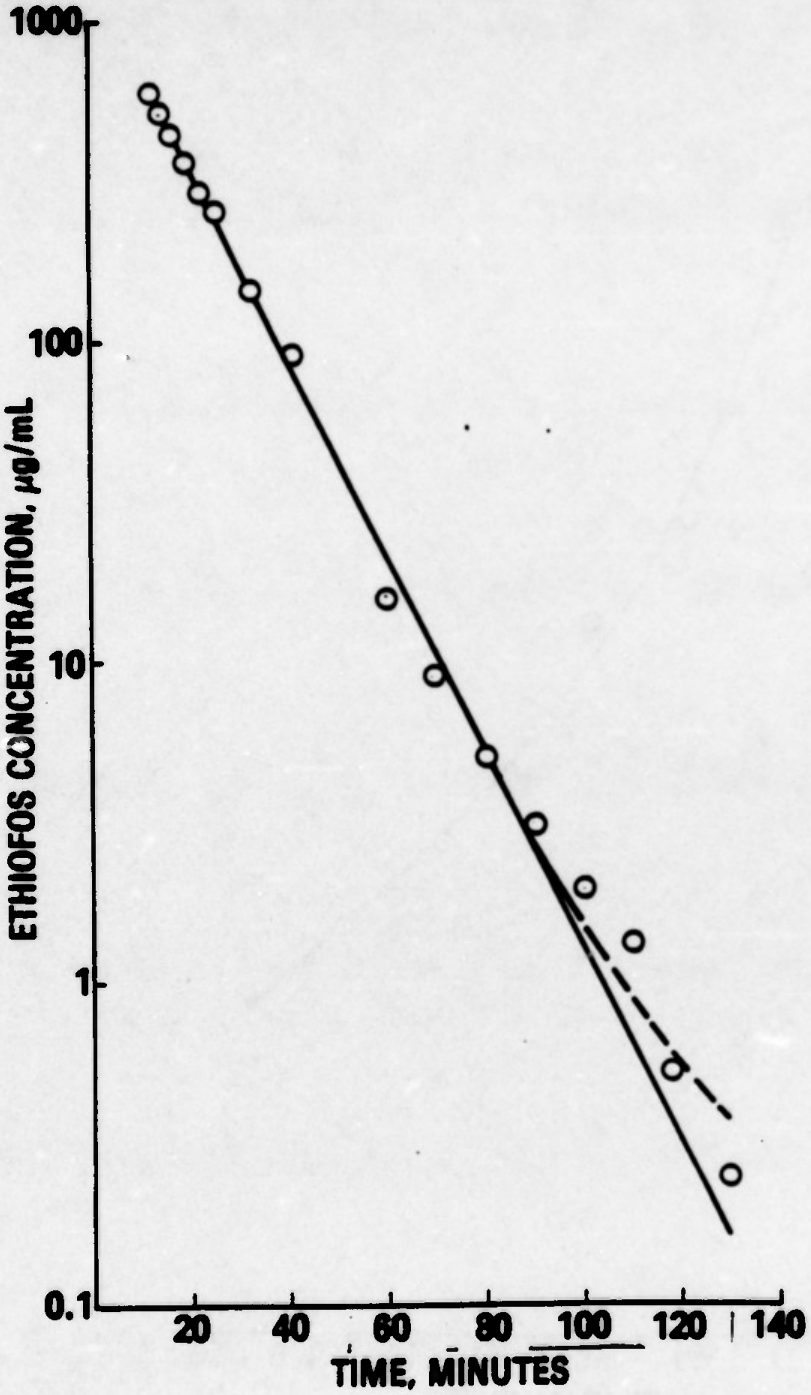


Figure 15. Ethiofos Pharmacokinetics--Dosing Study 10. The solid line is the concentration-time profile predicted by the one-compartment model. The dashed line is the portion of the concentration-time profile predicted by the two-compartment model that differs from that predicted by the one-compartment model.

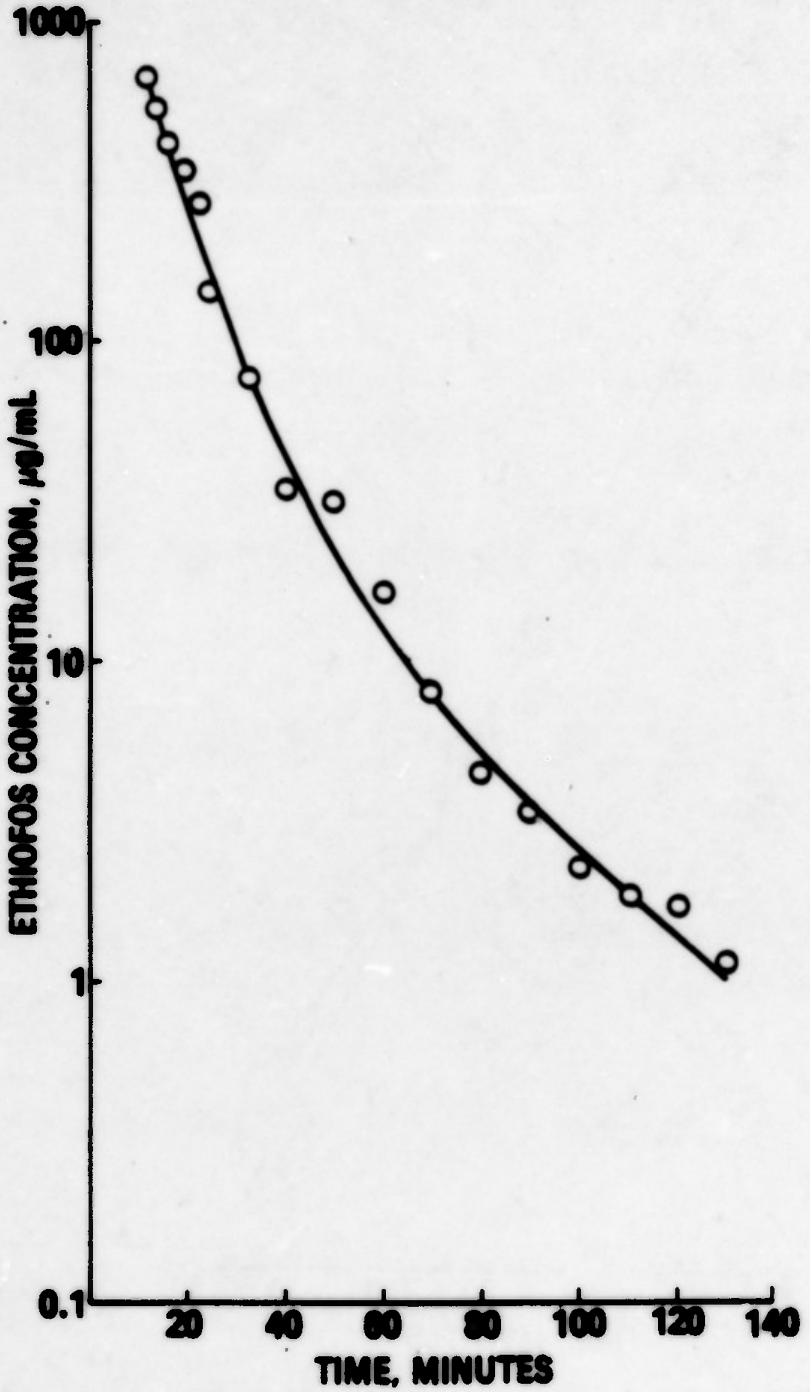


Figure 16. Ethiofos Pharmacokinetics--Dosing Study 14. The solid line is the concentration-time profile predicted by the two-compartment model.

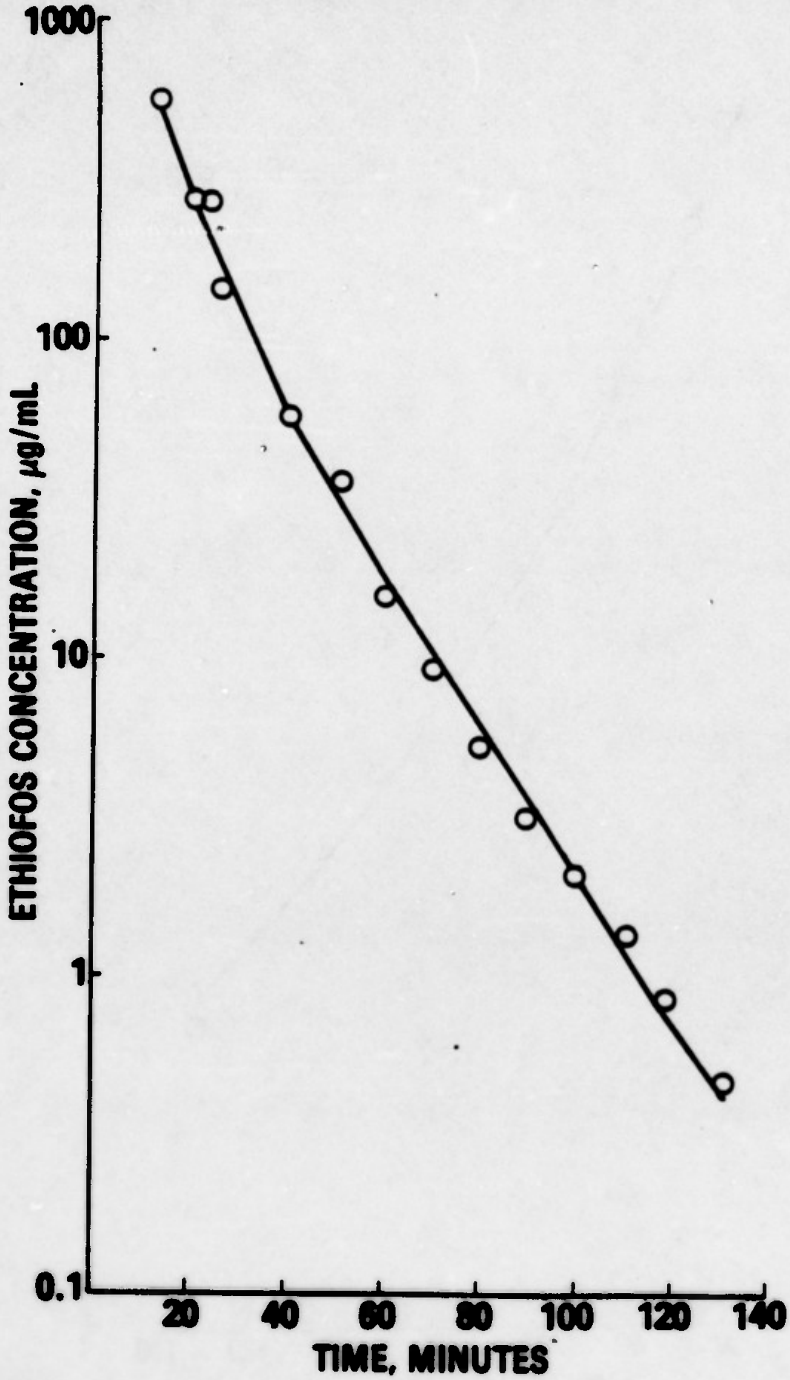


Figure 17. Ethiofos Pharmacokinetics--Dosing Study 17. The solid line is the concentration-time profile predicted by the two-compartment model.

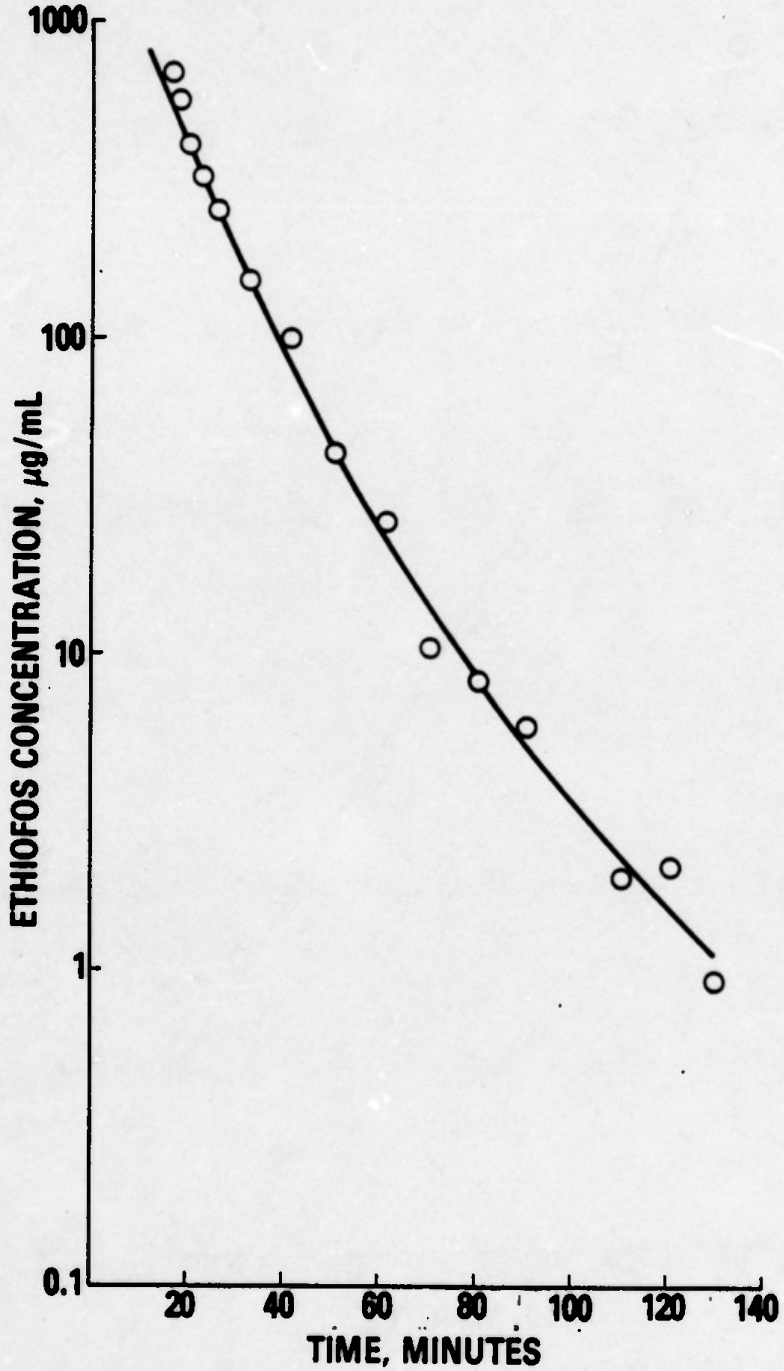


Figure 18. Ethiofos Pharmacokinetics--Dosing Study 18. The solid line is the concentration-time profile predicted by the two-compartment model.

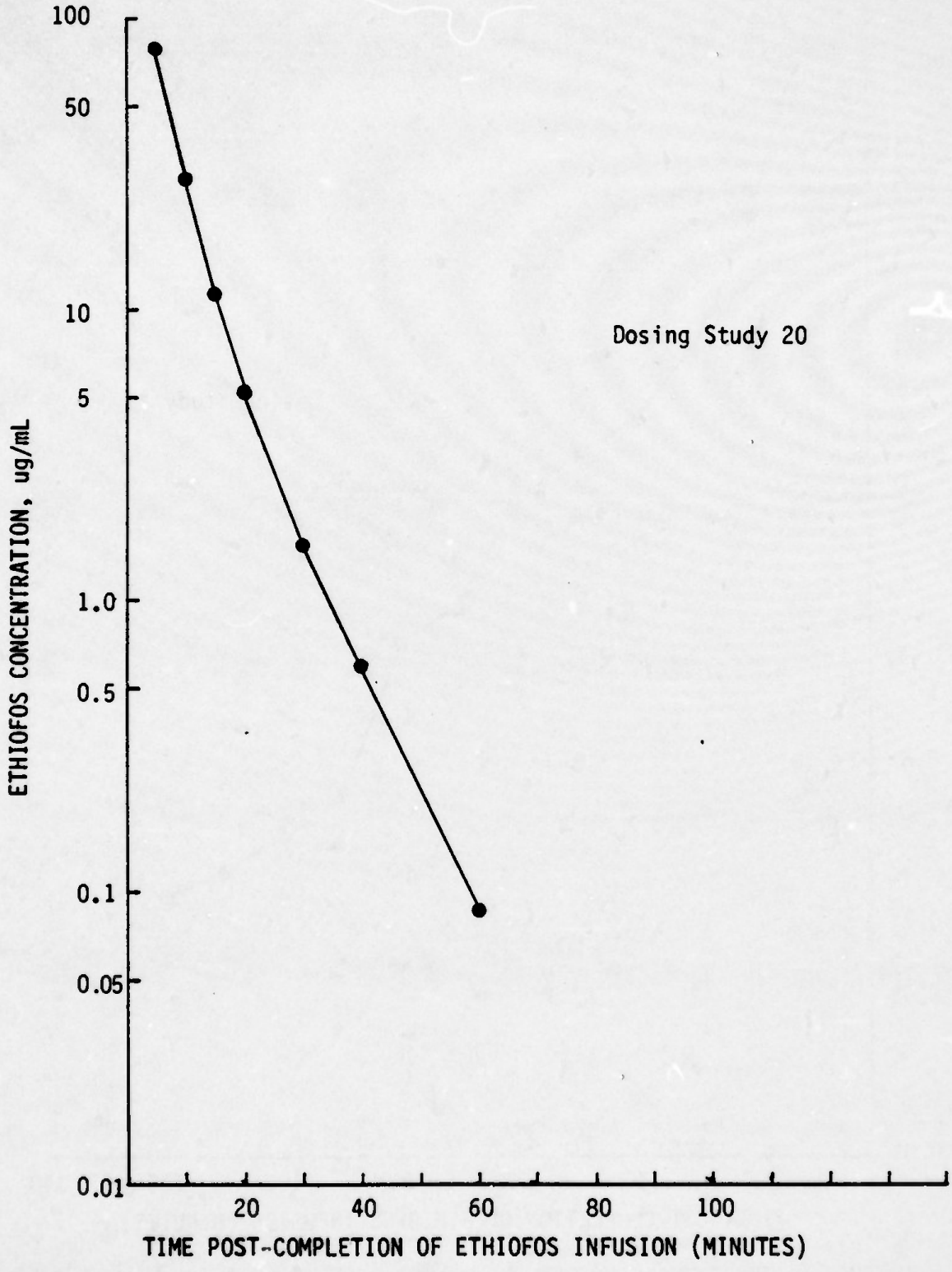


Figure 19. Plasma Ethiofos Levels Following 10-Minute IV Infusion of a Rhesus Monkey, Drug Dose of 150 mg/kg

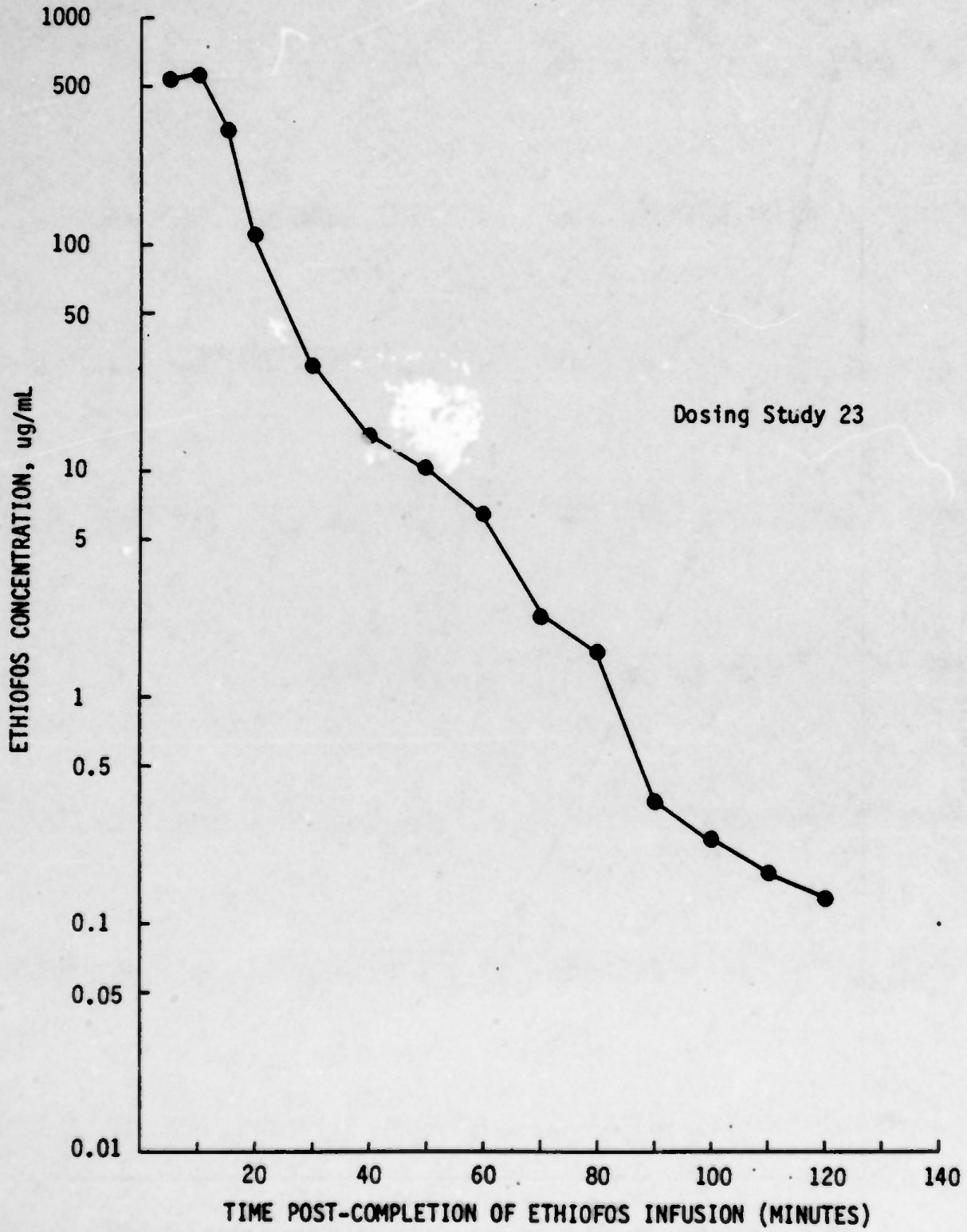


Figure 20. Plasma Ethiofos Levels Following 10-Minute IV Infusion of a Rhesus Monkey, Drug Dose of 200 mg/kg

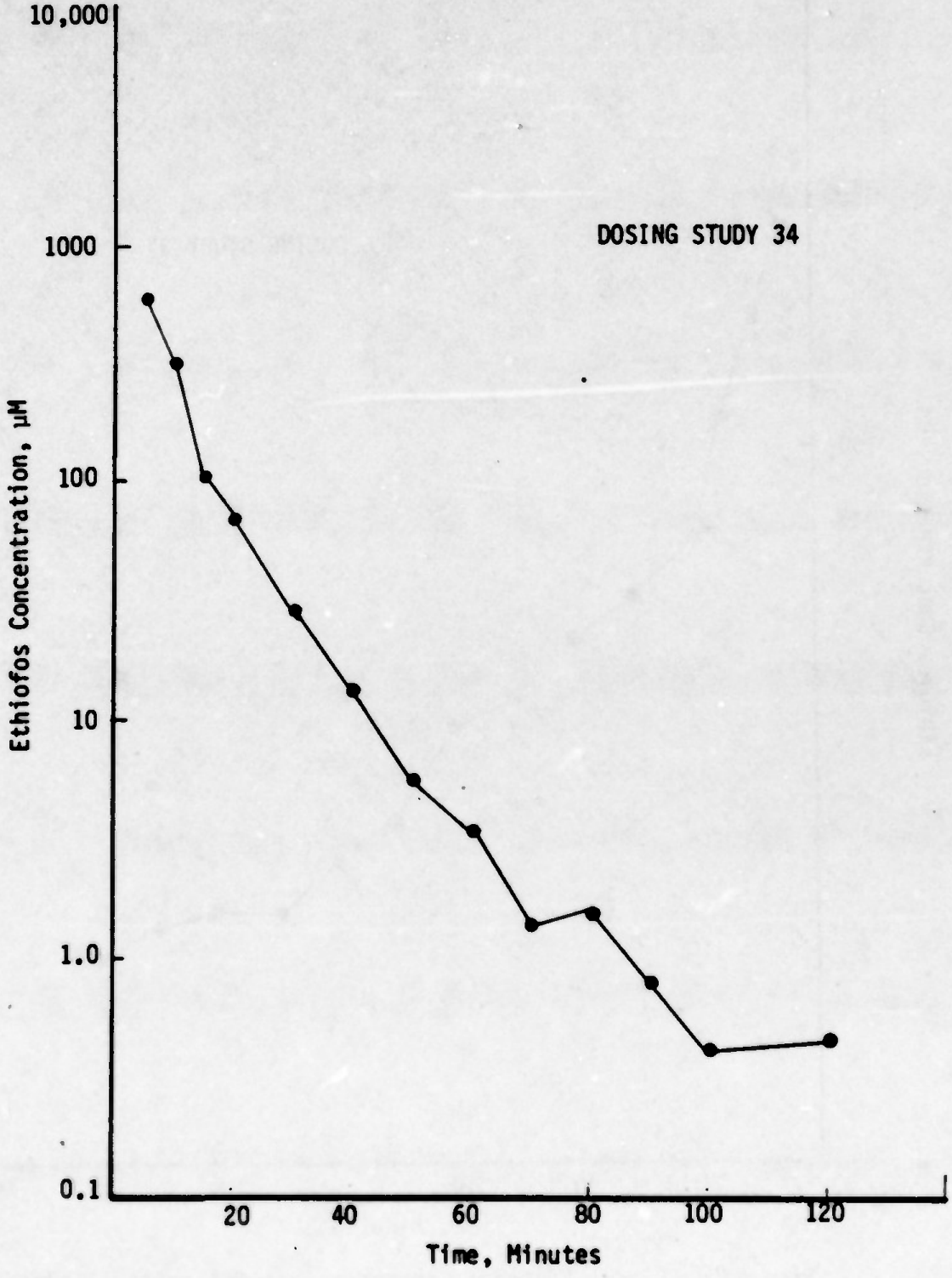


Figure 21. Plasma Ethiofos Concentrations Following IV Administration to a Rhesus monkey (150 mg/kg)

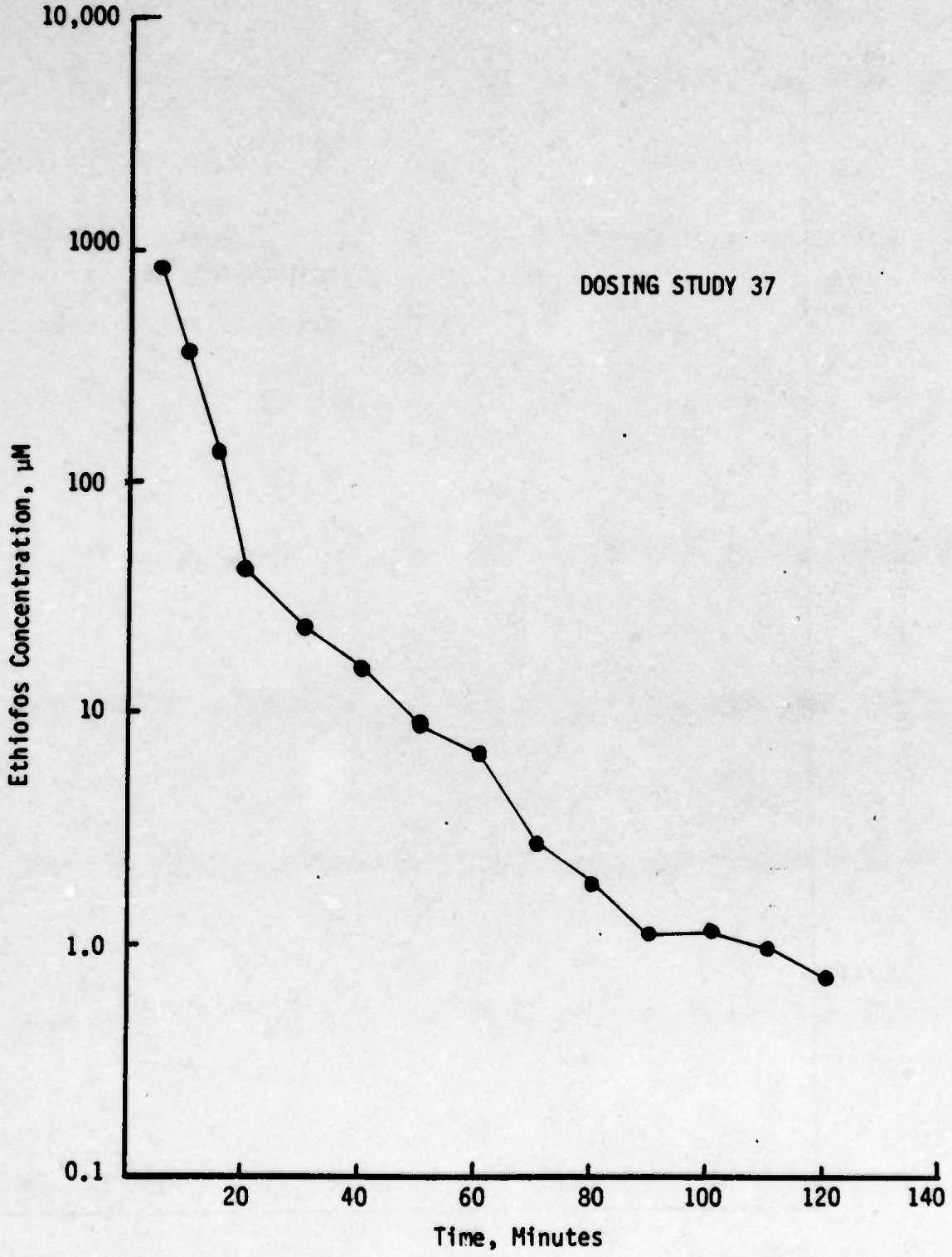


Figure 22 Plasma Ethiofos Concentrations Following IV Administration to a Rhesus Monkey (120 mg/kg)

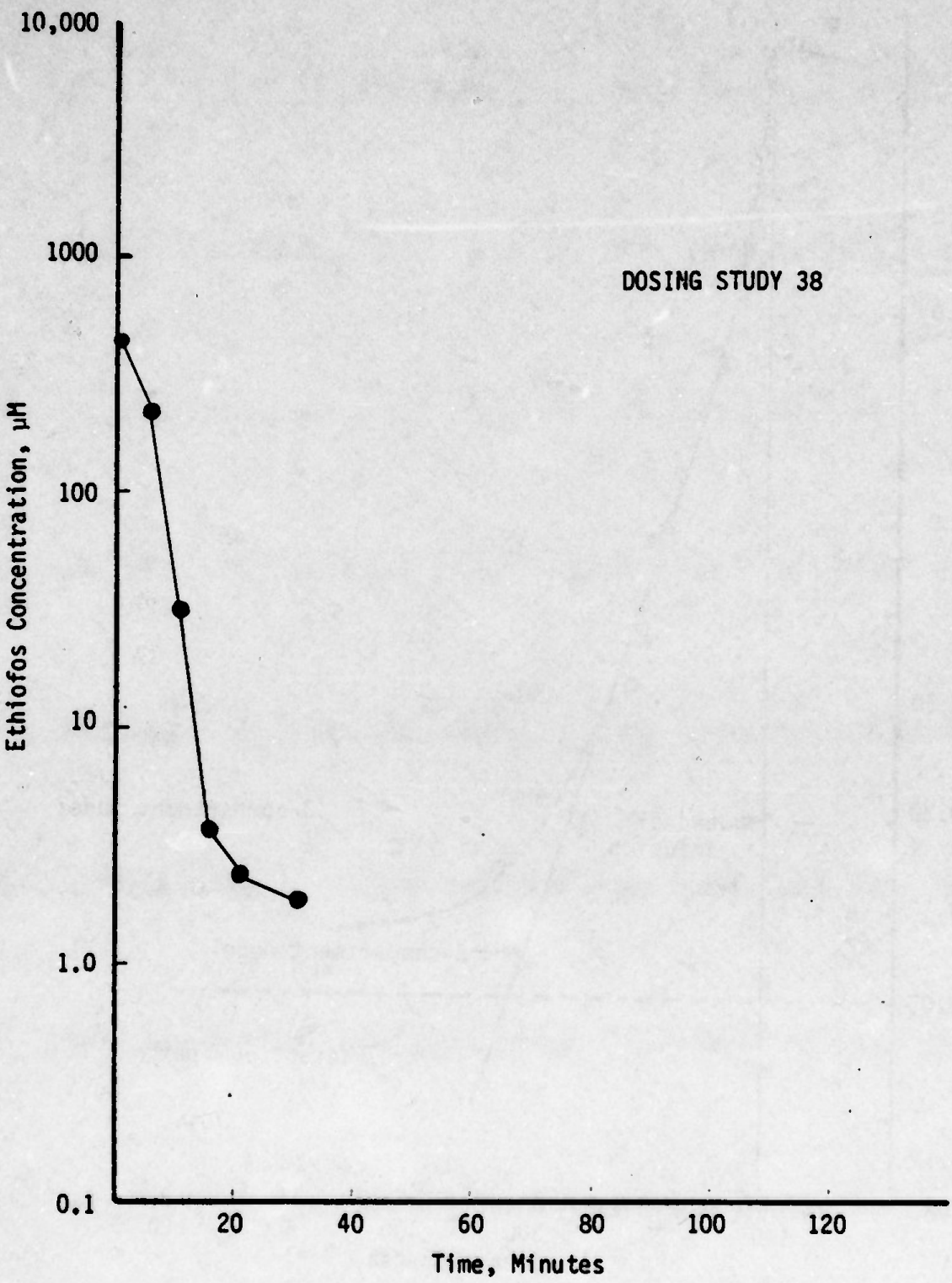


Figure 23. Plasma Ethiofos Concentrations Following IV Administration to a Rhesus Monkey (120 mg/kg)

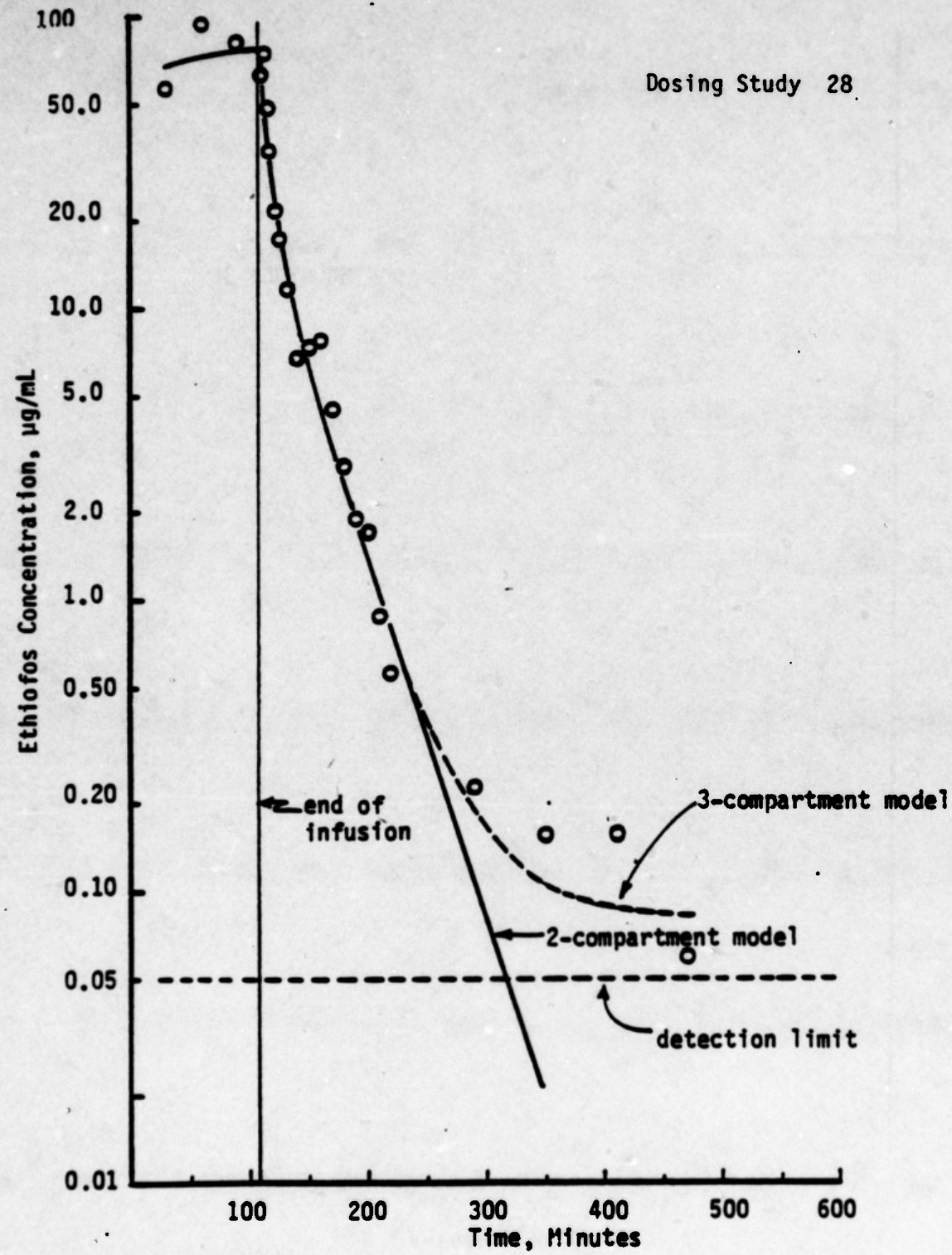


Figure 24. Concentration-Time Profile Following IV Administration of Ethiofos to a Beagle Dog. Dose of 150 mg/kg, infusion time of 110 minutes

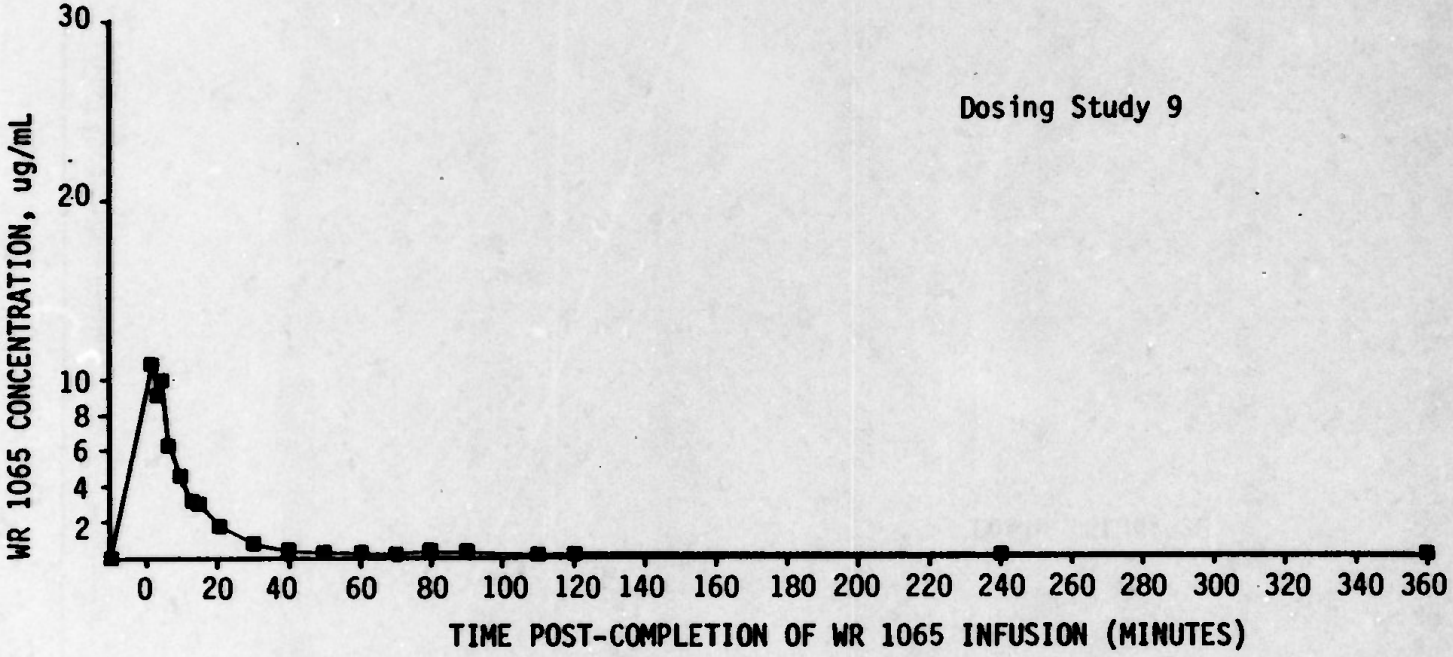


Figure 25. WR 1065 Plasma Concentration Observed in the Beagle Dog After an IV Infusion over 10 Minutes of a 60 mg/kg WR 1065 Dose

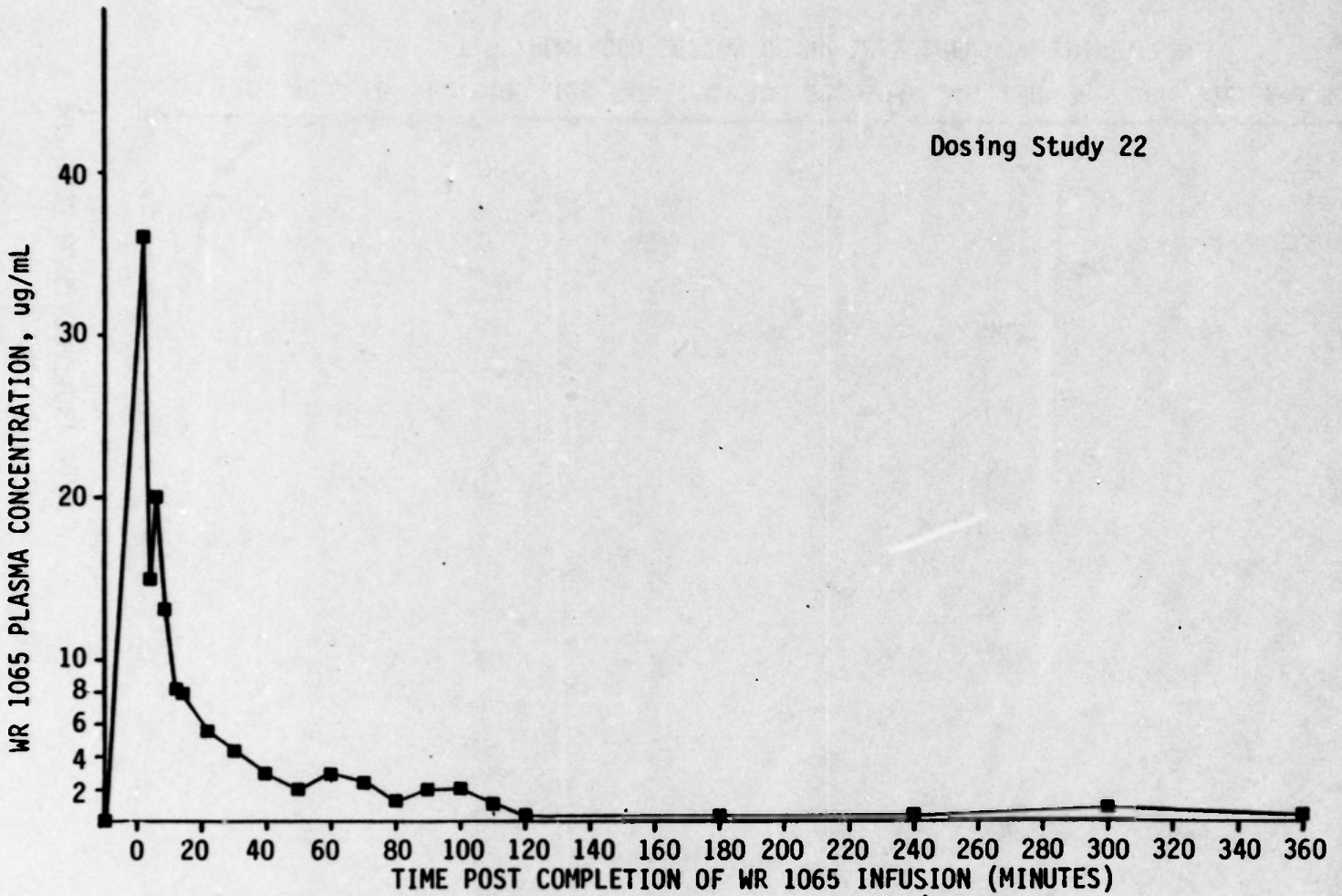


Figure 26. WR 1065 Plasma Concentrations Observed in the Beagle Dog After IV Infusion Over 10 Minutes of a 60 mg/kg WR 1065 Dose

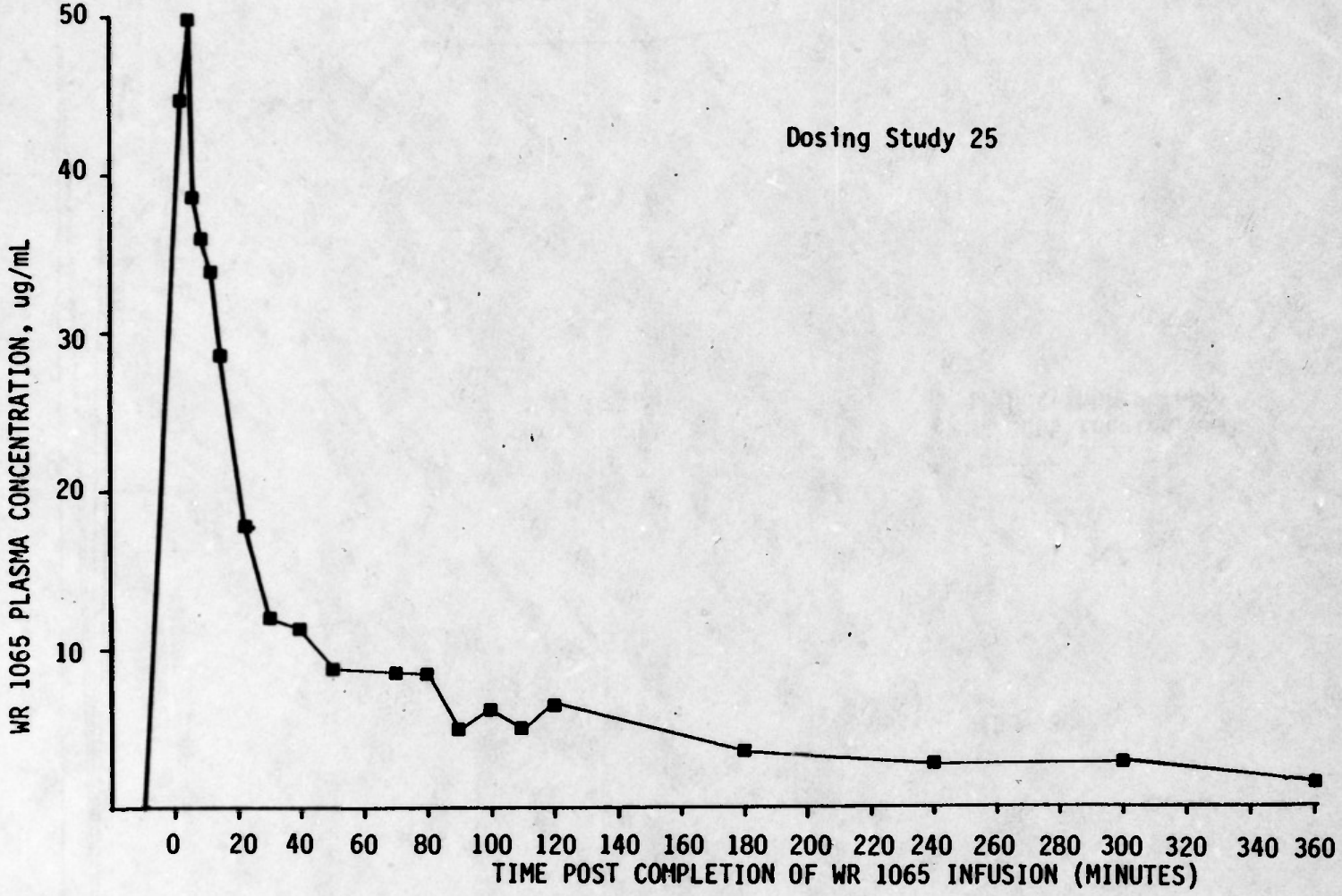


Figure 27. WR 1065 Plasma Concentrations Observed in the Beagle Dog After an IV Infusion Over 10 Minutes of a 60 mg/kg WR 1065 Dose (Dosing Study 25)

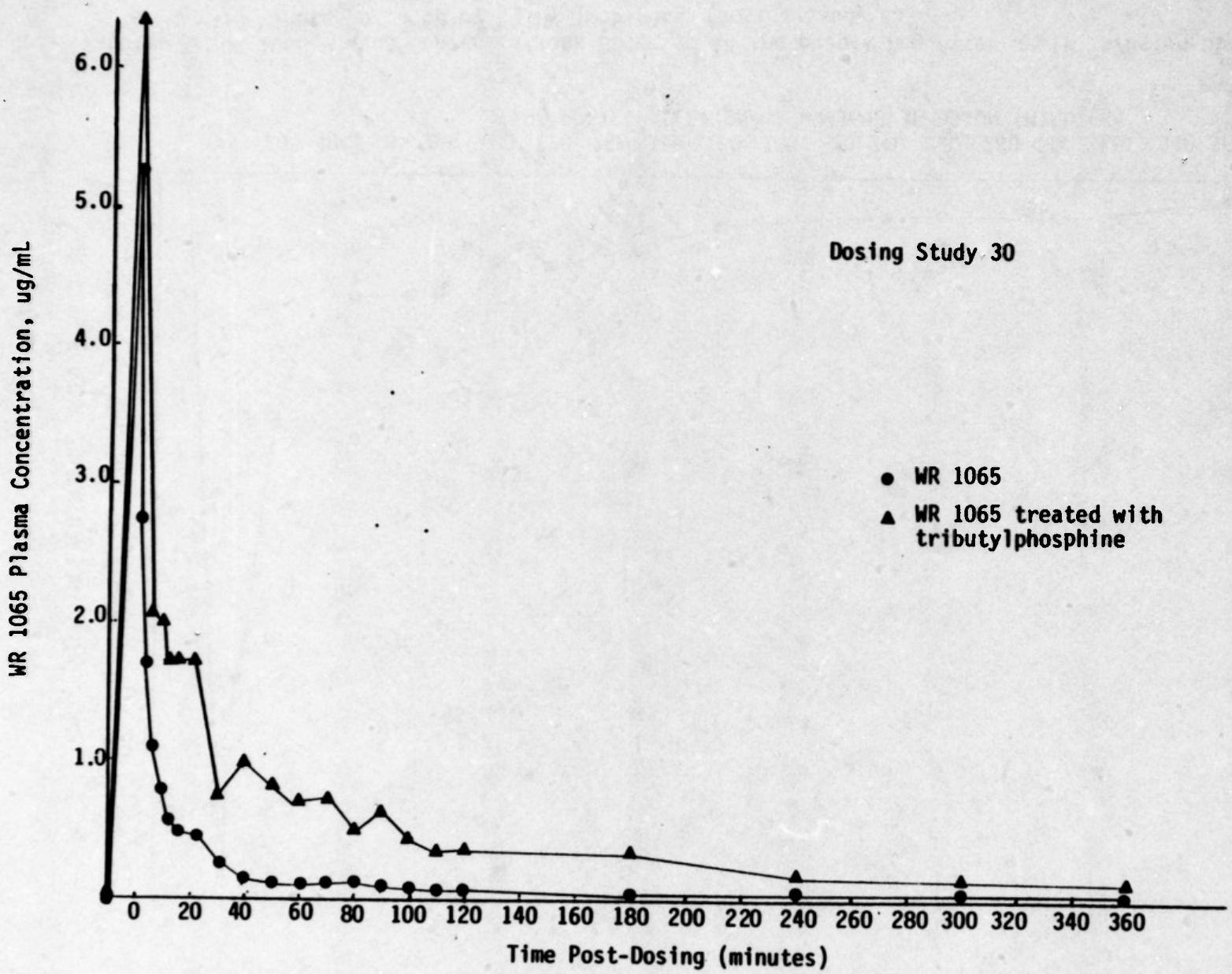


Figure 28. WR 1065 plasma concentrations observed after a 10 minute IV infusion of WR 1065 (60 mg/kg).

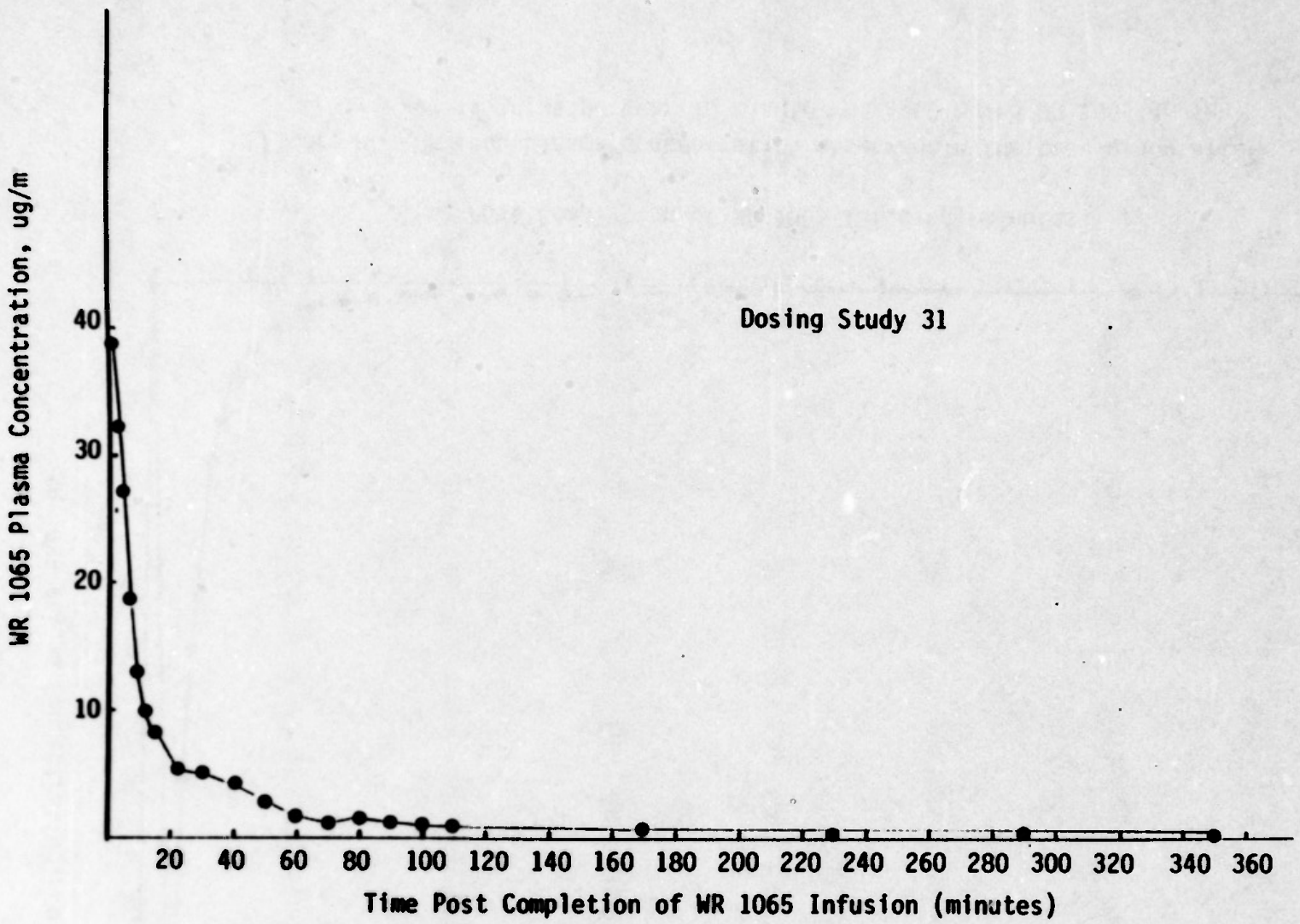


Figure 29. WR 1065 plasma concentration observed in the beagle dog after an IV infusion of a 60 mg/kg WR 1065 dose.

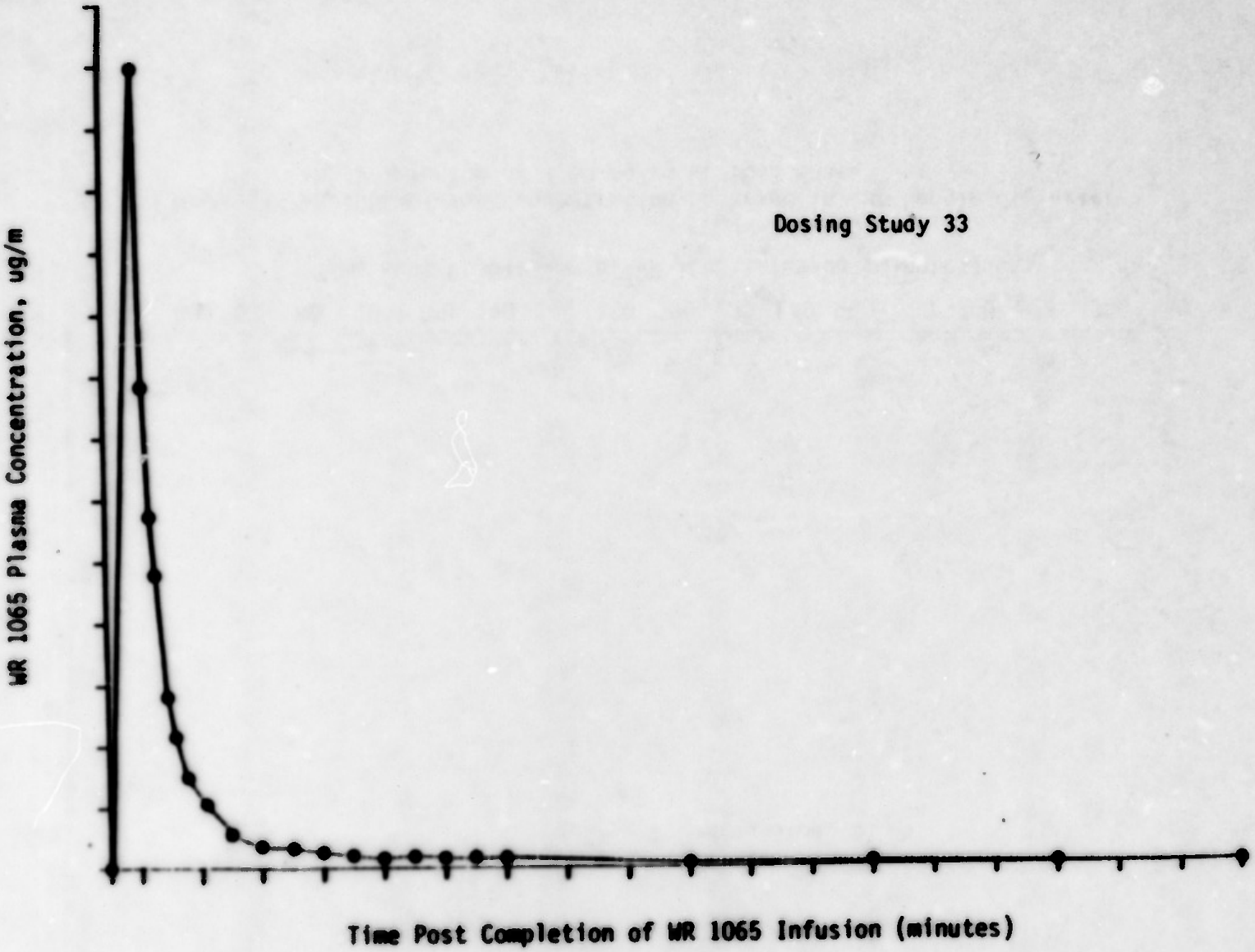


Figure 30. WR 1065 plasma concentration observed in the beagle dog after an IV infusion over 10 minutes of a 60 mg/kg WR 1065 dosing.

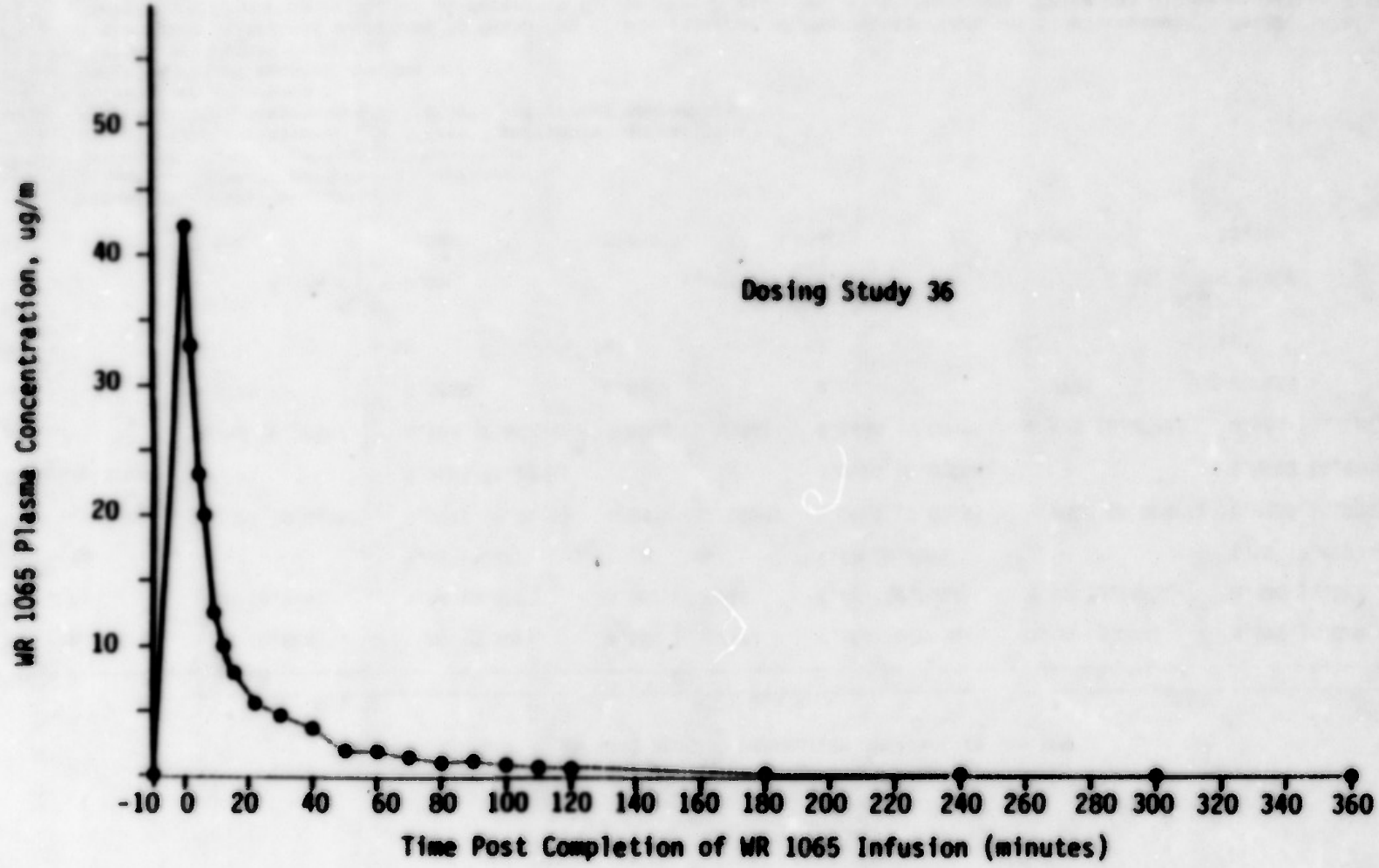


Figure 31. WR 1065 plasma concentration observed in the beagle dog after IV Infusion over 10 minutes of a 60 mg/kg WR 1065 dosing.

TABLE 9. SUMMARY OF WR 1065 KINETIC PARAMETERS OBTAINED IN THE DOG

Model	Dog 1		Dog 2		Dog 3	
	2	3	2	3	2	3
VC L/kg	1.16 (0.103)	1.09 (0.106)	0.636 (0.0578)	0.426 (0.0383)	1.05 (0.074)	0.930 (0.070)
VP1 L/kg	1.73 (0.248)	1.08 (0.481)	0.966 (0.178)	0.257 (0.0414)	1.63 (0.153)	0.846 (0.216)
VP2 L/kg	-	2.62 (1.27)	-	1.38 (0.308)	-	1.90 (0.410)
CLD1 L/min/kg	0.0630 (0.00620)	0.0572 (0.0136)	0.0301 (0.00329)	0.0421 (0.0113)	0.0600 (0.00474)	0.0555 (0.00650)
CLD2 L/min/kg	-	0.0203 (0.0106)	-	0.0178 (0.00235)	-	0.0202 (0.00606)
CLE L/min/kg	0.112 (0.0036)	0.104 (0.00431)	0.0366 (0.0034)	0.0366 (0.0030)	0.113 (0.0275)	0.106 (0.0061)
WSS	0.5740	0.3663	0.5437	0.2379	0.2731	0.1284
DF	17	16	18	16	18	16
F	4.25 0.025 < P < 0.05		10.28 0.001 < P < 0.005		9.02 0.001 < P < 0.005	
AIC	-3.858	-3.080	-5.410	-19.58	-20.55	-33.16

VC - volume of central compartment
VP1 - volume of "fast" peripheral compartment
VP2 - volume of "slow" peripheral compartment
CLD1 - distributional clearance for "fast" peripheral compartment
CLD2 - distributional clearance for "slow" peripheral compartment
CLE - elimination clearance
WSS - weighted sum of squared residuals
DF - degrees of freedom
F - F-statistic calculated according to Boxenbaum et al. (Journal of Pharmacokinetics and Biopharmaceutics 2:123, 1974)
AIC - Akaike information criterion as described by Yamaoka et al. (Journal of Pharmacokinetics and Biopharmaceutics 6:165-175, 1978)

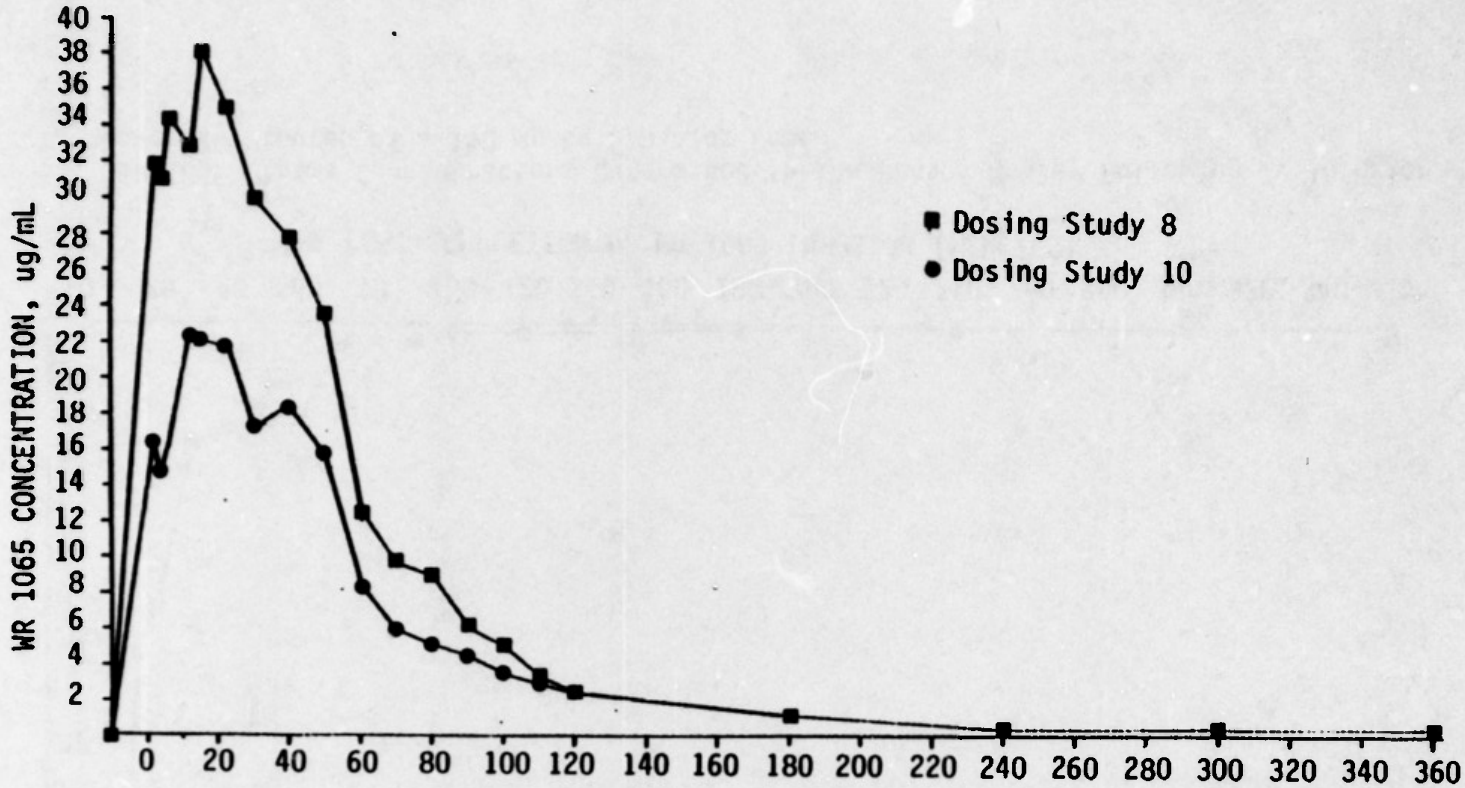


Figure 32. WR 1065 Plasma Concentrations Following a 10-Minute IV Infusion of Ethiofos (150 mg/kg) to a Beagle Dog

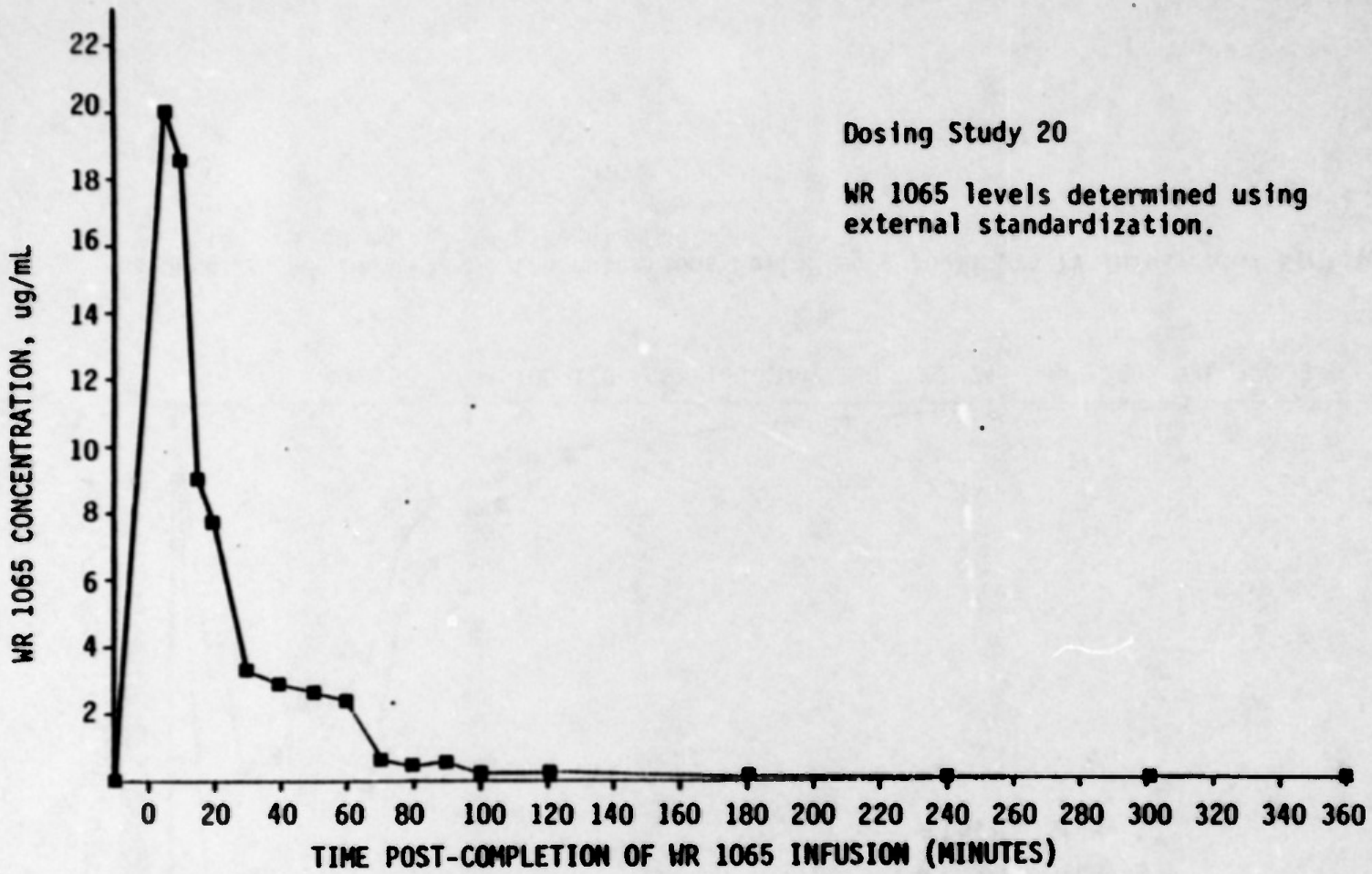


Figure 33. WR 1065 Plasma Concentrations Determined in the Rhesus Monkey Following IV Infusion over 10 Minutes of a 150 mg/kg Ethiofos Dose

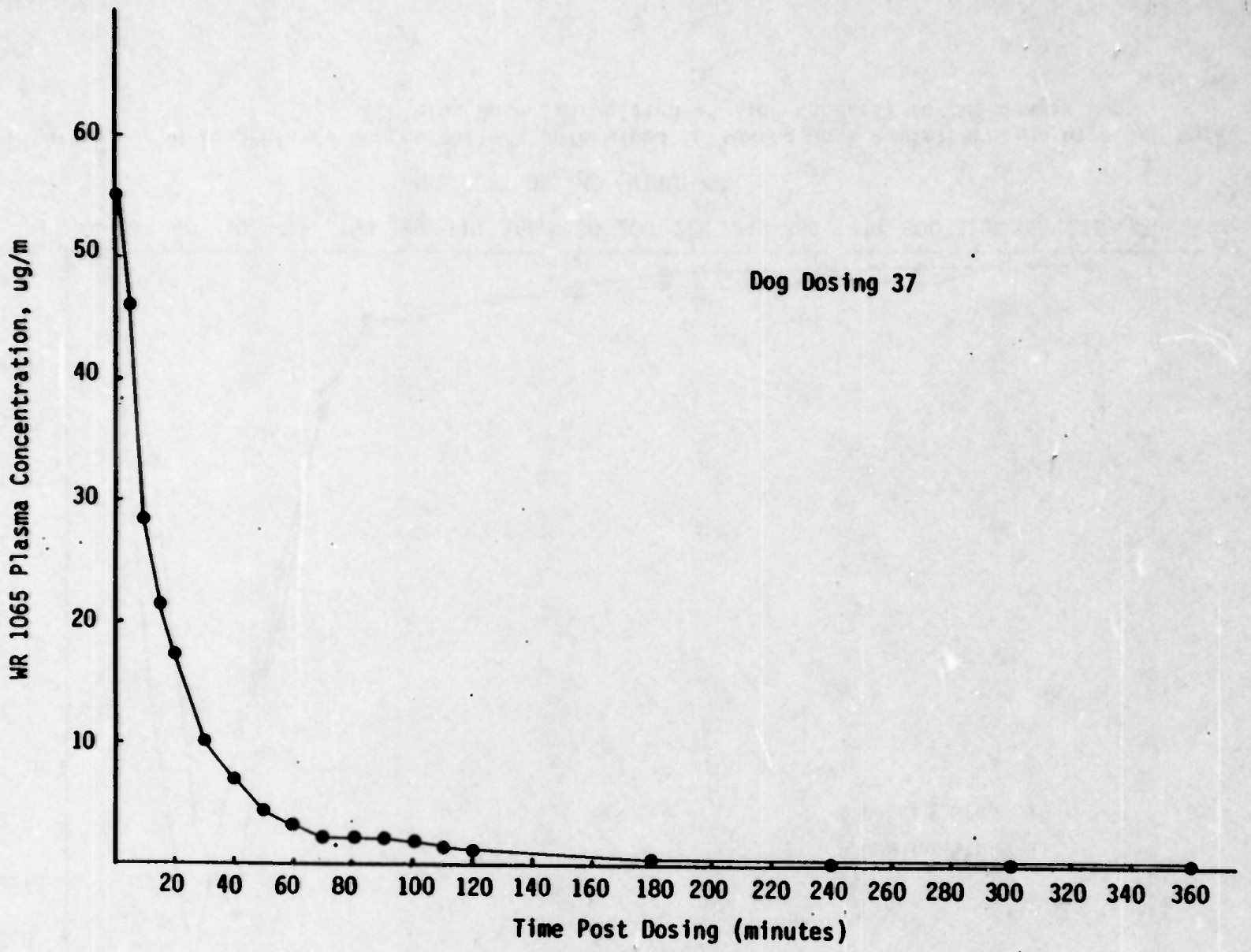


Figure 34. WR 1065 plasma concentration observation in monkey after 10 minute IV infusion of ethiofos (150 mg/kg).

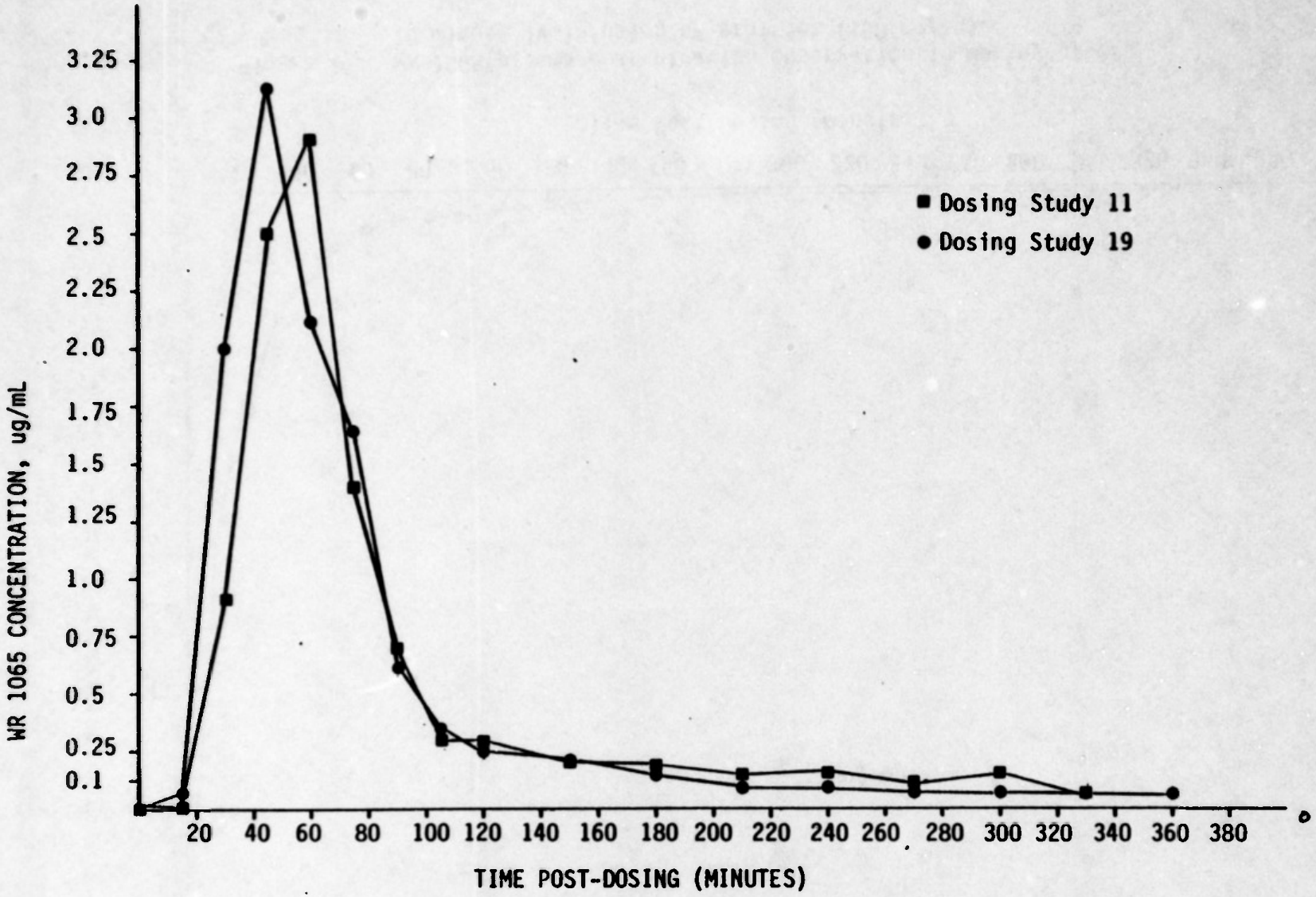


Figure 35. WR 1065 Plasma Concentrations Determined Following Oral Administration of a 150 mg/kg Ethiofos Dose (Formulated as Microspheres) to the Beagle Dog

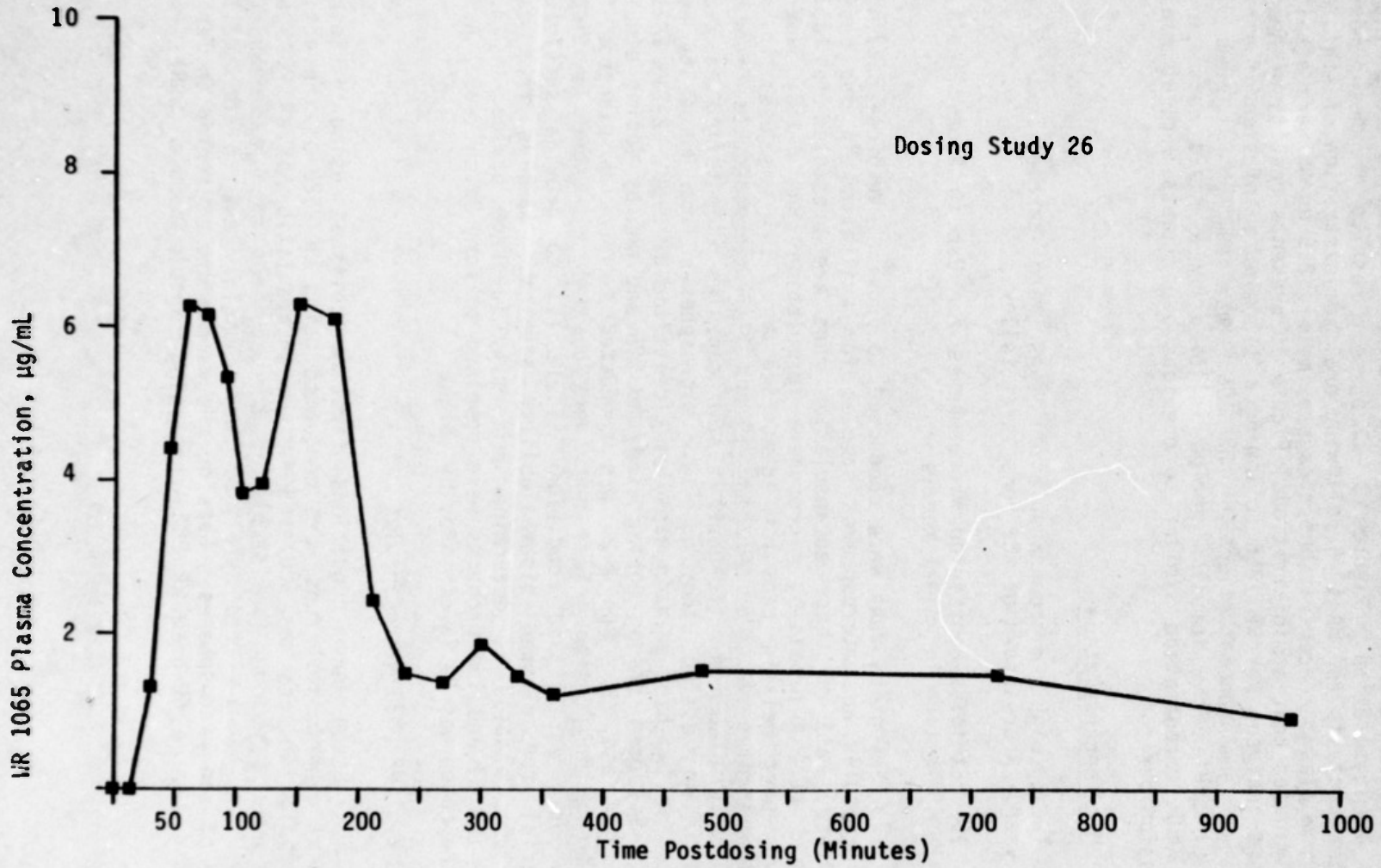


Figure 36. WR 1065 Plasma Concentration Observed in Beagle Dog after an Oral Dose of Ethiofos (150 mg/kg microspheres in gelatin capsules)

1065 following oral administrations of encapsulated ethiofos. Maximum plasma concentration levels of WR 1065 (3 µg/mL) occur 40 to 60 minutes post administration. Figures 37-39 present results of rhesus monkey plasma analysis for WR 1065 following oral administration of ethiofos. Maximum plasma concentrations of approximately 2.5 µg/mL were attained 180 minutes post administration. Figure 40 presents results of beagle plasma analyses for WR 1065 following a 110 minute infusion of ethiofos. Plasma concentrations of WR 1065 increase to a maximum (20 µg/mL) during the infusion period followed by a rapid decrease in WR 1065 concentration similar to previous experiments with 10 minute infusion periods.

C. Microencapsulation

Twenty-eight microencapsulation runs were carried out during the year. A breakdown of the runs is as follows.

1. Ethiofos-Containing Microspheres for Use in Bioavailability Testing in Animal Models

Seventeen runs were conducted to provide ethiofos-containing microcapsules and microspheres samples for evaluation in dog dosing studies. Data on these encapsulation runs are presented in Tables 10 and 11. A promising microsphere formulation (Run 2-16B; Samples in two-part gelatin capsules identified as 2-19) was used in dog dosing studies nos. 11, 12, 13, 19 and 21. Microcapsules from run 2-23 were placed in two-part gelatin capsules (identified as 2-27) used in dog dosing study no. 16. Microspheres from Run 2-16A were placed in two-part gelatin capsules (identified as 2-30, 2-31, 2-31A, and 2-34) used in dog dosing study no. 26 and monkey dosing studies nos. 24 and 29. Run 2-24 was conducted to provide placebos for a control dog dosing. Five runs were carried out under identical, previously established conditions (Table 11) to provide sufficient material for planned bioavailability studies. Samples from each run were assayed to determine uniformity (average payload = 24.7, CV = 0.16%) and the products were combined to form Batch 2-39. Assay of the batch was 24.7% ethiofos by weight.

2. WR 2823 Microcapsules

Based upon conditions first established using ethiofos, several experimental runs were conducted using WR 2823 as the active material. Success was achieved in the encapsulation of WR 2823 but the product was not as stable as the ethiofos capsules under the conditions of the acid hyrolysis test, possibly due to the smaller size of the microspheres. Data on the sample are presented in Tables 12 and 13. A 60-gram WR 2823-containing capsule sample (SwRI 7-595

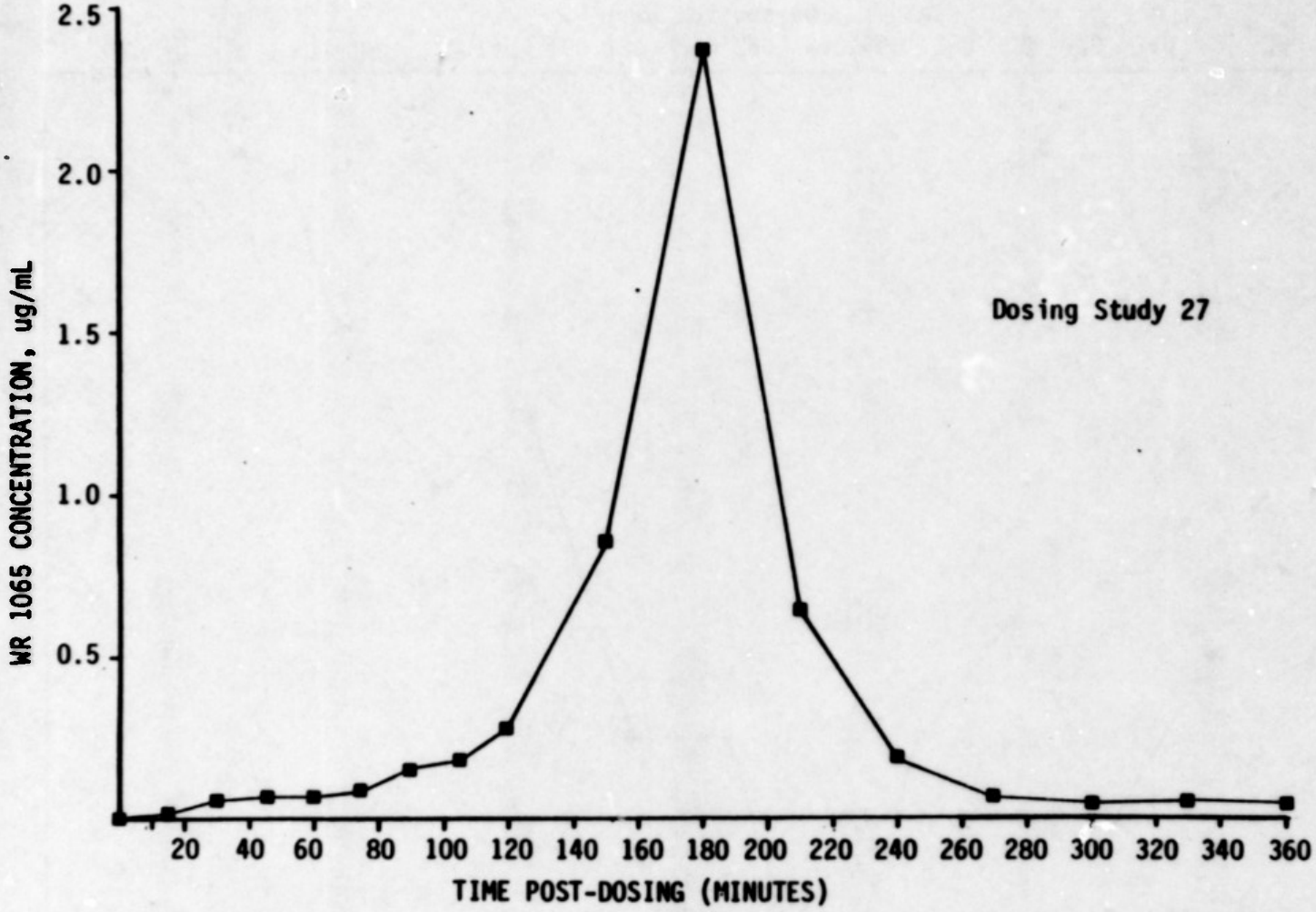


Figure 37. WR 1065 Plasma Concentrations Observed in the Monkey After an Oral Dose of Ethiofos (150 mg/kg in gelatin capsules)

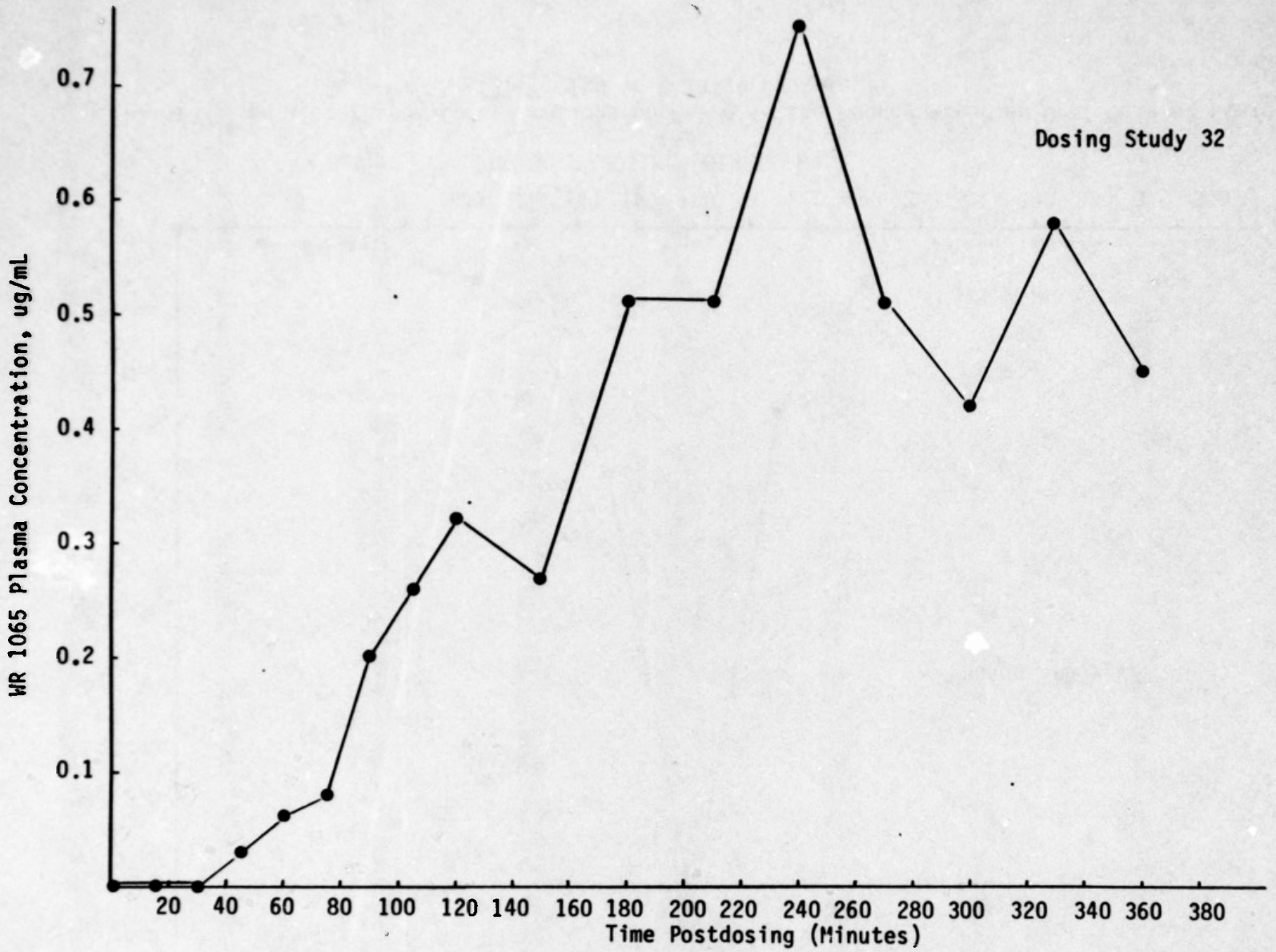


Figure 38. WR 1065 Plasma Concentration Observed in Monkey after Oral Dose of Ethiofos (200 mg/kg in Gelatin Capsules)

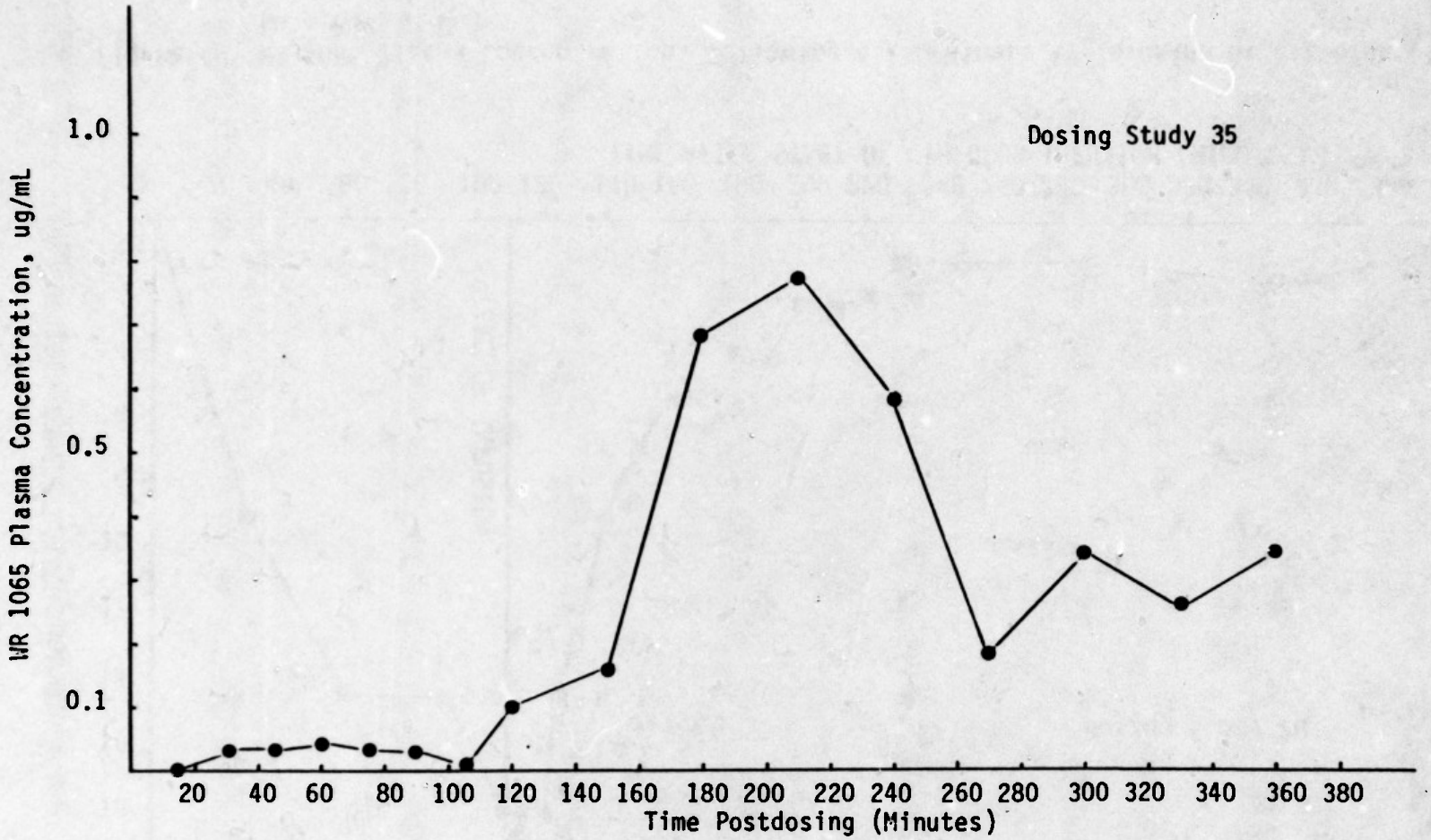


Figure 39. WR 1065 Plasma Concentration Observed in the Monkey after an Oral Dose of Ethiofos (400 mg/kg gelatin capsules)

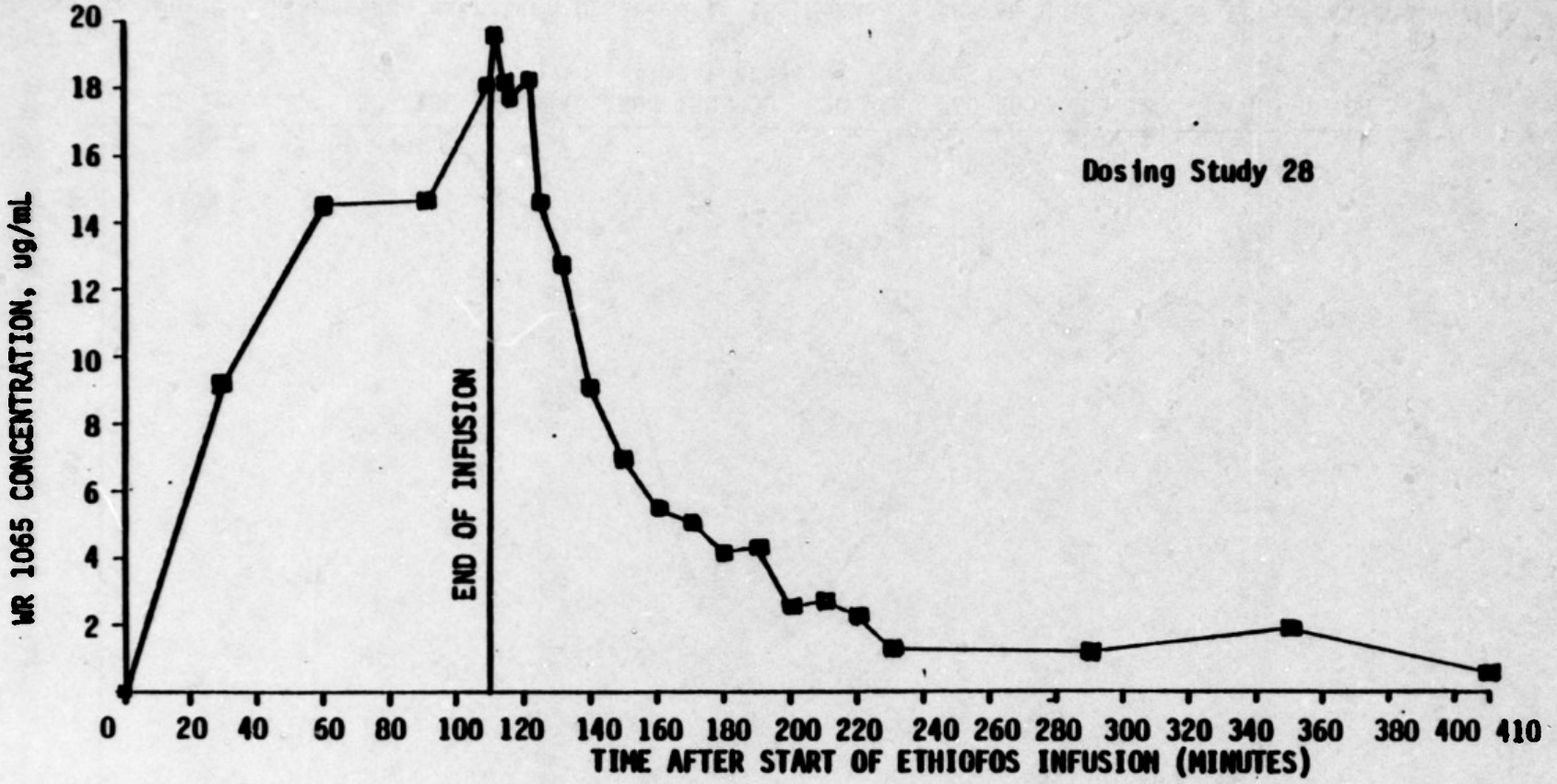


Figure 40. WR 1065 Plasma Concentrations Following a 110-Minute IV Infusion of Ethiofos (150 mg/kg) to a Beagle Dog

TABLE 10. ENCAPSULATION DATA ON ETHIOFOS MICROCAPSULES[a,b]

Run No.	Shell Composition (% by wt)	Fill Composition (% by wt)	Feed Rate (g/m) Shell	Feed Rate (g/m) Fill	Head Speed (RPM)	Temperature of System (°C)	Theoretical % Ethiofos (Anhydrous)	Analysis % Ethiofos (Anhydrous)	Capsule Size (μ)	Comment
2-7A	50% Emersol 6349 stearic acid 50% Grocol 600-E triglyceride	40% Emersol 6349 stearic acid 40% Grocol 600-E triglyceride 20% Ethiofos	9.0	23.0	-1800	-68.3	11.5	11.2	425-600	Satisfactory product
2-13A	"	"	12.5	23.3	-2080	-68.3	10.4	10.0	425-600	Satisfactory product
2-22	50% Emersol 6349 stearic acid 50% Grocol 600-E triglyceride	40% Emersol 6349 stearic acid 20% Grocol 600-E triglyceride 20% Ethiofos	10.0	23.0	-1800	68.3	11.1	9.8	600-710	Made well
2-23	80% Emersol 6349 stearic acid 20% Grocol 600-E triglyceride	62% Emersol 6349 stearic acid 16% Grocol 600-E triglyceride 20% Ethiofos	10.0	23.0	-1800	68.3	11.1	11.2	600-710	Made well
2-24 (placebo)	"	—	23.0	-	-1550	68.3	-	-	600-710	Made well
2-25	"	64% Emersol 6349 stearic acid 16% Grocol 600-E triglyceride 20% Ethiofos	10.7	23.0	-1800	68.3	10.8	9.8	600-710	Made well

a. Nozzle Size Used: 0.020" inside, 0.040" outside diameter, 12 mil spacing.
b. Ethiofos dispersed in fill material. Mixture kept under nitrogen blanket.

TABLE 11. ENCAPSULATION DATA ON ETHIOFOS MICROSPHERES[a]

Run No.	Matrix Composition (% by wt)[b] Ethiofos	Emerzol 8340	Brocol 800-E	Feed Rate (g/m)	Head Speed (RPM)	Temperature of System (°C)	Theoretical % Ethiofos (Anhydrous)	Analysis % Ethiofos (Anhydrous)	Size (u) Distribution (%) 400-800	800-710	<400>800 or <800>800	Comment
2-13B	25	60	15	18.4	~2000	~65.0	20	18.50	77.0		23.0	Made well
2-13C	20	64	16	18.4	~2000	~65.0	16	16.00	76.0		24.0	Made well
2-16A[c]	30	56	14	20.0	~1800	~60.3	24	22.30		78.0	22.0	Made well
2-16B[c]	30	56	14	20.0	~1800	~60.3	24	23.75		78.0	21.0	Made well
2-16C[d]	30	56	14	20.0	~1800	~60.3	24	22.00		80.0	80.0	Matrix mixture was viscous
2-16D[d]	30	56	14	20.0	~1800	~60.3	24	22.40		78.0	22.0	Matrix mixture was slightly viscous
2-25[c]	30	56	14	20.0	~1800	~60.3	24	24.00		80.0	20.0	Made well
2-30[e]	30	56	14	20	1800	60	24	24.00		78.5	22.5	Made well

a. Nozzle Size Used: 0.033" diameter
b. Ethiofos dispersed in matrix material. Mixture was kept under nitrogen blanket
c. Ethiofos (<106 u) was hand milled and sieved
d. Ethiofos (<106 u) was mechanically milled and sieved
e. Composed of five runs

TABLE 12. ENCAPSULATION DATA ON WR 2823 MICROCAPSULES[a,b]

SwRI Sample No [Run No.]	Shell Composition [% by wt]	Fill Composition [% by wt]	Feed Rate [g/m] Shell	Feed Rate [g/m] Fill	Head Speed [RPM]	Temperature of System [°C]	Theoretical % WR 2823 (Anhydrous)	Analysis % WR 2823 (Anhydrous)	Capsule Size [u]	Sample Wt [grams]
7-586 [2-17A]	50% Emersol 6348 stearic acid 50% Sreeol 880-E triglyceride	40% Emersol 6346 stearic acid 40% Sreeol 880-E triglyceride 20% WR 2823, Lot ASA7886874 [<100 u, hand milled and sieved]	12.5	23.3	~2000	~60.3	11.3	9.0	400-600	60

a. Nozzle Size Used: 0.020" inside, 0.040" outside diameter, 10 mils spacing
b. WR 2823 dispersed in fill material. Mixture kept under nitrogen blanket.

TABLE 13. WR 2823 MICROCAPSULE ANALYSES, HYDROLYSIS (pH 1.0), AND SYNTHETIC INTESTINAL FLUID (pH 7.5) TEST RESULTS

			Assay[c] - pH 1.0 Time (min)			Assay[c] - pH 7.5 Time (min)			
SmRI Sample No. (Run No.)	Theoretical[a] Payload %	Capsule[b] Assay %	30 (% Retained)	90 (% Retained)	90 (% Retained)	15 (% Released)	30 (% Released)	60 (% Released)	120 (% Released)
7-685 (2-17A)	11.3	9.9	5.6 (56.6)	3.9 (39.4)	3.8 (38.4)	1.0 (89.9)	0.2 (98.0)	0.1 (99.0)	0.1 (99.0)

a. Gravimetric composition of melt prior to microcapsule production.

b. Determined by HPLC analysis, average of two determinations.

c. Determined by HPLC analysis of recovered microcapsules, average of two determinations.

(2-17A)) was submitted on February 23, 1984 to Walter Reed Army Institute of Research for evaluation.

3. Improved Formulations

Ten runs were made with varied matrix components to produce formulations with improved properties. The goals were: (1) to improve stability in acidic media, (2) improve shell integrity after accelerated aging tests, (3) increase drug release characteristics near neutral pH and (4) increase absorption of the drug by adding promoters to the formulation. Four of the runs were "placebo" runs to investigate encapsulation conditions and properties of the new excipients. The remainder contained ethiofos and the products are currently being analyzed and evaluated. A synopsis of the runs is presented in Table 14. Run 2-41 was conducted to study the use of Grocol 600-[illegible] as a fill matrix and Run 2-42 to study Polyglycol E-4000 for the same use. Run 2-43 was conducted to study the use of a promoter, oleic acid, as a fill matrix with the product microcapsules to be coated at a later time with an enteric shell; however, no microcapsules were produced due to operation difficulties.

Runs 2-44A, B, C and D were conducted to produce placebos using oleic acid and soybean oil as promoters and fill matrix materials.

Runs 2-45A and B were conducted to determine the use of a promoter, soybean oil, as a matrix fill material with the product microcapsules to be coated at a later time with an enteric shell.

Run 2-46 was conducted using a similar shell formulation; however, no quality microcapsules were recovered.

4. Stability Testing

Hydrolytic stability and in vitro release results on these microspheres are presented in Table 15. Hydrolytic stability testing on microspheres from Runs 2-16A, 2-16B, 2-33 and Batch 2-39 indicated that 92 to 100% of the original ethiofos remained after 90 minutes at pH 1. In vitro release data on Runs 2-16A, 2-16B, 2-33 and Batch 2-39 capsule samples indicated 96-98% of the encapsulated ethiofos was released in the synthetic intestinal fluid (pH 7.5) within 120 minutes. Procedures for assaying the microspheres, conducting the hydrolytic stability tests, and determining the release rates are presented in Appendices C, D, and E, respectively. Additional details of the samples used in the oral studies are presented in Table 16.

TABLE 14. ENCAPSULATION ON RECENT EXPERIMENTAL MICROCAPSULE FORMULATIONS[a,b]

Run No.	Shell Composition (% by wt)	Fill Composition (% by wt)	Feed Rate (g/s) Shell	Feed Rate (g/s) Fill	Head Speed (RPM)	Temperature of System (°C)[c]	Theoretical % Ethiofos (Anhydrous)	Analysis % Ethiofos (Anhydrous)	Capsule Size (μ)	Comment
2-41	91.0% Emersol 6348 20.0% Grocol 600-E	70% Grocol 600-E 30% Ethiofos	11.0	21.6	1800	F—68-69 S—68-72	15.9	14.9	500-710	Microcapsules were produced
2-42	80.0% Emersol 6342 20.0% Grocol 600-E	70% Polyglycol E-4000 30% Ethiofos	11.0	21.6	1800	F—68-72 S—71-77	15.9	14.5	"	Microcapsules were produced
2-43	70.2% Gelatin 150 Bloom Type A 29.8% Sorbitol USP	70% Oleic acid 30% Ethiofos	29.0	15.2	1800	F—RT S—80-88	-	-	-	Operational difficulties, no microcapsules produced
2-44A	70.2% Gelatin 150 Bloom Type A 29.8% Sorbitol USP	100% Soybean oil	36.6	12.6	1800	F—RT S—80-84	-	-	250-850	Placebo run
2-44B	94.3% Gelatin 150 Bloom Type A 29.8% Sorbital USP	100% Oleic acid	36.6	12.6	1800	"	-	-	"	Placebo run
2-44C	94.3% Gelatin 150 Bloom Type A 5.7% HWS	100% Oleic acid	36.6	10.0	1800	"	-	-	"	Placebo run
2-44D	94.3% Gelatin 150 Bloom Type A 5.7% HWS	100% Soybean oil	36.6	10.0	1800	"	-	-	"	Placebo run
2-45A	70.2% Gelatin 150 Bloom Type A 29.8% Sorbitol USP	70% Soybean oil 30% Ethiofos	36.6	11.0	1800	F—RT S—80-83	12.3	10.0	"	To be coated with an enteric shell
2-45B	94.3% Gelatin 150 Bloom Type A 5.7% HWS	70% Soybean oil 30% Ethiofos	36.6	9.6	1800	"	15.0	11.6	"	To be coated with an enteric shell
2-46	91.7% Gelatin 300 Bloom Type A 8.3% HWS	70% Soybean oil 30% Ethiofos	36.6	9.6	625	F—RT S—80-83	17.1	-	-	Microcapsules leaked on standing. No sample.

a. Nozzle Size Used: 0.020" inside, 0.040" outside diameter, 20 mil spacing.
b. Ethiofos dispersed in fill material. Mixture kept under nitrogen blanket.
c. F - fill; S - shell.

TABLE 15. ETHIOFOS MICROCAPSULE ANALYSES, HYDROLYSIS (pH 1.0), AND SYNTHETIC INTESTINAL FLUID (pH 7.5) TEST RESULTS

Run No.	Theoretical[a] Payload %	Capsule[b] Assay %	Assay[c] - pH 1.0 Time (min) 90 (% Retained)	Assay[c] - pH 7.5 Time (min) 15 (% Released)	30 (% Released)	60 (% Released)	120 (% Released)
2-7A	11.5	11.2	8.5 (75.9)	d	d	d	d
2-13A	10.4	10.0	9.8 (98.0)	d	d	d	d
2-13B	20.0	18.5	14.1 (76.2)	d	d	d	d
2-13C	16.0	18.5	14.0 (75.7)	d	d	d	d
2-16A	24.0	22.3	20.9 (92.4)	0.5 (97.8)	0.2 (99.1)	0.2 (99.1)	0.1 (99.5)
2-16B	24.0	23.9	23.2 (97.5)	1.0 (95.8)	0.3 (98.7)	0.2 (95.8)	0.2 (99.2)
2-16C	24.0	22.5	15.9 (70.7)	0.7 (96.9)	0.2 (99.2)	0.2 (99.2)	0 (100.0)
2-16D	24.0	22.5	17.9 (79.8)	0.5 (97.8)	0.2 (99.1)	0.2 (99.1)	0 (100.0)
2-22	11.0	9.9	7.7 (77.7)	3.0 (68.7)	1.2 (87.9)	0.5 (94.9)	0.1 (99.0)
2-23	11.1	11.2	10.8 (94.8)	0.6 (94.8)	0.1 (99.1)	0.1 (99.1)	0 (100.0)
2-25	10.9	9.8	9.3 (94.8)	1.2 (87.7)	0.2 (98.0)	0.1 (99.0)	0 (100.0)
2-33	30	24.8	24.8 (100.0)	0.8 (96.4)	0.7 (97.2)	0.3 (98.8)	0.5 (98.0)
2-41	15.9	14.4	0.5 (3.5)	d	d	d	d
2-42	15.9	15.7	0.8 (3.8)	d	d	d	d

a. Gravimetric composition of melt prior to micro[capsule/sphere] production.
b. Determined by HPLC analysis, average of two determinations.
c. Determined by HPLC analysis of recovered microcapsules, average of two determinations.
d. Not determined.

TABLE 16

ORAL DOSAGE SAMPLES

Dose Study No.	Capsule Dosage Form	Wt of Micro(spheres/ capsules) g	Wt of Ethiofos (Anhydrous) g	Dosage Level mg ethiofos/kg
11	4x2-19[a]	8.90	2.11	150
12	2x2-19[a] 1x2-19A[b]	4.90	1.16	75
13	4x2-19[a]	8.90	2.11	150
16	5x2-27[c]	10.13	1.13	75
19	4x2-19[a] 1x2-19A[a]	9.35	2.22	150
21*	4x2-19[a]	8.90	2.11	150
24**	4x2-30[b]	3.20	0.71	200
26*	4x2-31[c] 1x2-31A[d]	9.35	2.08	150
27**	1x2-32[e]	0.73***	0.58	150
29**	4x2-30[b] 1x2-34[f]	3.41	0.76	200

* Dog dosings
** Monkey dosings
*** Neat ethiofos

TABLE 16. (continued)

a. 2-19 two-part gelatin capsules composed of 2-16B (500-710 μ) 23.75% ethiofos approximately 2.2250 g each capsule. Gelatin capsule--No. J104-1/8 oz from South Texas Serum Co.

b. 2-19A two-part gelatin capsules composed of 2-16B (500-710 μ) 23.75% ethiofos approximately 0.4500 g each capsule. Gelatin capsule--NDC 0002-2413-02, No. 00 7PL17A, SF 2135 AMS from Lilly.

c. 2-27 two-part gelatin capsules composed of 2.23 (500-710 μ) 11.15% ethiofos approximately 2.0250 each capsule. Gelatin capsule--No. J104-1/8 oz from South Texas Serum Co.

d. 2-30 two-part gelatin capsules composed of 2-16A (500-710 μ) 22.35% ethiofos approximately 0.8000 g each capsule. Gelatin capsule--NDC 0002-2414-02, No. 000 from Lilly.

e. 2-31 two-part gelatin capsules composed of 2-16A (500-710 μ) 22.35% ethiofos approximately 2.2250 each capsule. Gelatin capsule--No. J104-1/8 oz from South Texas Serum Co.

f. 2-31A two-part gelatin capsules composed of 2-16A (500-710 μ) 22.3% ethiofos approximately 0.4500 g each capsule. Gelatin capsule--NDC 0002-2413-02, No. 00 7PL17A, SF2135 AMS from Lilly.

g. 2-23 two-part gelatin capsule composed of 0.73 g of ethiofos WR 2721 AX BK 02762 PV-V-116. Gelatin capsule--NDC 0002-2414-02, No. 000 from Lilly.

h. 2-34 two-part gelatin capsule composed of 2-16A (500-710 μ) 22.3% ethiofos approximately 0.2100 g each capsule. Gelatin capsule--NDC 0002-2414-02, No. 000 from Lilly.

An experiment to simulate aging of the formulations was begun. Capsule samples were placed in sealed glass vials in an air atmosphere and stored at room temperature (23° ± 1°C) and at elevated temperature (37°C). Samples are removed at selected times, assayed for ethiofos content, and evaluated for hydrolytic stability and release rate properties. Testing is complete on samples stored for one month. The data are presented in Table 17. Assay of ethiofos-containing microspheres from 2-16A, 2-16B, 2-16C, and 2-16D after storage for one month at both temperatures showed no loss of ethiofos. These results tend to disprove the hypothesis that low recovery of drug after acid stability testing of aged samples was due to reaction of drug and matrix. It is more likely that thermal aging produced a change in the integrity of the shell or matrix which gave poorer protection of the ethiofos.

Neat ethiofos trihydrate, aged one month under the test conditions of room temperature and 37°C, showed no loss of activity, i.e, HPLC analysis for anhydrous ethiofos did not change. However, drug samples stored at the higher temperature lost some weight, an observation attributed to changes in the extent of hydration.

Microspheres containing hand-milled ethiofos were found to be more stable in the acid stability tests than those containing mechanically-milled drug. Results obtained from testing the milled materials with an electron scanning microscope showed that mechanical milling produced much smaller particles than hand milling. The greater surface area of the smaller particles is believed to be responsible for the lower stability (greater reactivity).

5. Analysis of Encapsulated Ethiofos Recovered During Dog Dosing

In Study No. 11 the dog vomited at 39, 48, 53, 59, 63 and 68 minutes, post administration. The emesis was labeled Vomit 1 through 6 and assay of the samples is presented in Table 18. About 19 percent of the ethiofos dose was found in the liquid vomit and recovered microspheres. However, in Study No. 12, when the dose administered was reduced fifty percent, only 0.1 percent of the ethiofos was found in the vomit. Vomiting, which occurred at 49, 65 and 75 minutes, post administration, was much smaller in volume.

TABLE 17. STORAGE STABILITY TEST RESULTS (1 MONTH)
Ethicfce microcapsule analyses, hydrolysis (ph 1.0), and synthetic intestinal fluid (ph 7.5) test results

Run No.	Theoretical[a] Payload %	Capsule[b] Assay %	Assay[c] - pH 1.0 Time (min) 60 (% Retained)	Assay[c] - pH 7.5 Time (min) 15 (% Released)	30 (% Released)	60 (% Released)	120 (% Released)
2-13B	20						
Original analysis[e]		18.5	14.1 (75.2)	d	d	d	d
Room temp[f]		15.7	8.5 (54.1)	3.2 (79.8)	0 (100.0)	0.5 (96.8)	0 (100.0)
Elevated at 37°C[f]		20.5	1.1 (5.3)	2.4 (89.3)	0.5 (97.5)	1.5 (96.7)	1.5 (92.7)
2-13C	15						
Original analysis		15.5	14.0 (75.7)	d	d	d	d
Room temp		14.4	13.5 (93.8)	2.1 (86.4)	1.4 (90.3)	0 (100.0)	0 (100.0)
Elevated at 37°C		11.8	0.2 (1.7)	0.8 (92.4)	0 (100.0)	0 (100.0)	0 (100.0)
2-16A	24						
Original analysis		22.3	20.5 (92.4)	2.2 (90.1)	0.9 (96.0)	0.9 (96.0)	0.4 (98.2)
Room temp		25.0	19.5 (78.2)	2.0 (92.0)	0.4 (96.4)	0.4 (96.4)	0 (100.0)
Elevated at 37°C		23.5	0.2 (0.9)	3.0 (87.1)	1.5 (94.4)	0.5 (97.9)	0.5 (97.9)
2-16B	24						
Original analysis		23.8	23.2 (97.5)	4.2 (82.4)	1.5 (94.5)	0.9 (96.2)	0.9 (96.2)
Room temp		23.5	20.5 (86.9)	11.8 (49.5)	1.5 (94.5)	0.4 (96.3)	0.4 (96.3)
Elevated at 37°C		20.8	1.1 (5.3)	2.4 (88.5)	0 (100.0)	0.5 (97.8)	0 (100.0)
2-16C	24						
Original analysis		22.5	15.9 (70.7)	0.7 (96.9)	0.2 (99.1)	0.2 (99.1)	0 (100.0)
Room temp		24.5	22.8 (93.1)	1.9 (92.2)	0.2 (99.2)	0.4 (98.4)	0.2 (99.2)
Elevated at 37°C		23.1	0.4 (1.7)	0.3 (96.7)	0.2 (99.1)	0.2 (99.1)	0 (100.0)
2-16D	24						
Original analysis		22.4	17.9 (78.8)	0.5 (97.8)	0.2 (99.1)	0.2 (99.1)	0 (100.0)
Room temp		23.8	21.3 (89.5)	0.2 (99.2)	0.4 (98.3)	0.4 (98.3)	0.5 (98.7)
Elevated at 37°c		22.7	0.5 (2.2)	0.9 (96.0)	0.8 (96.5)	0.2 (99.1)	0.3 (98.7)
2-23	11.1						
Original analysis		11.2	10.5 (94.5)	0.5 (94.8)	0.1 (99.1)	0.06(99.5)	0 (100.0)
Room temp		15.5	14.0 (89.7)	1.4 (81.0)	0.2 (98.7)	0.2 (98.7)	0.1 (99.4)
Elevated at 37°C		15.4	2.0 (15.0)	0.4 (97.4)	0 (100.0)	0.1 (99.4)	0 (100.0)
2-25	10.9						
Original analysis		9.8	9.3 (94.8)	1.2 (87.8)	0.3 (96.9)	0.1 (99.0)	0 (100.0)
Room temp		14.2	12.7 (89.4)	1.1 (92.3)	0.1 (99.5)	0.2 (98.8)	0 (100.0)
Elevated at 37°C		14.5	8.0 (41.1)	1.0 (93.2)	0.2 (99.8)	0.3 (97.9)	0.1 (99.5)

a. Gravimetric composition of melt prior to microcapsule production.
b. Determined by HPLC analysis, average of two determinations.
c. Determined by HPLC analysis of recovered microcapsules, average of two determinations.
d. Not determined.
e. Assay of capsules at zero time.
f. Assay of capsules after 1 month at test conditions used.

TABLE 18. ASSAY OF VOMIT FROM DOG DOSING STUDIES

Dose Study No.	Capsule Dosage Form	Wt of Microspheres (g)	Wt of Ethiofos (Anhydrous) (g)	Dosage Level mg Ethiofos/kg	Vomit: Liquid Ethiofos Equiv (g)	Vomit: Microspheres Ethiofos Equiv (g)	Vomit: Total Ethiofos (g)	Vomit: Percent Ethiofos Lost
11	4x2-18[a]	8.9	2.11	150	0.02348	0.38564	0.40912	19.4
12	2x2-18 1x2-18A[b]	4.9	1.18	75	0.00059	0.00027	0.00086	0.07

a. 2-18 two-part gelatin capsules composed of 2-168 (500-710 microns) 23.75% Ethiofos
Approximately 2.2250 g each capsule
Gelatin capsule--No. J104-1/8 oz from South Texas Serum Co.

b. 2-18A two-part gelatin capsules composed of 2-168 (500-710 microns) 23.75% Ethiofos
Approximately 0.4500 g each capsule
Gelatin capsule--NDC 0002-2413-02, No. 00 7PL17A, SF2135 AMS

V. CONCLUSIONS

(1) Ethiofos in beagle plasma is stable for at least six months when the sample is stored at -75°C. Some decomposition occurs when the storage temperature is -20°C, the extent of decomposition being greater with low original concentrations (0.5-5 µg/mL).

(2) WR 1065 in beagle plasma decomposes rapidly. It is stabilized by the addition of 0.1M monochloroacetic acid/0.2M perchloric acid and storage at -75°C relative to WR 1729, an internal standard. After storage under these conditions for 47 days ratios of drug to internal standard are unchanged but 55% of the original amounts present decomposed.

(3) The plasma assay for ethiofos, in its present form, is suitable for use in pharmacokinetic studies. Acceptable accuracy and precision has been demonstrated down to about 0.1 µg/mL.

(4) The assay for WR 1065 is suitable for use in pharmacokinetic studies. Sensitivity, precision and accuracy were demonstrated to be acceptable in pilot dosing experiments with beagles and rhesus monkeys.

(5) Preliminary evaluations of the WR 33278 plasma assay indicate that the method will be satisfactory for conducting pharmacokinetic experiments.

(6) Although very low concentration levels of ethiofos were measured in plasma following oral administration of the microencapsulated drug, analysis of the plasma for "free" and covalently bound WR 1065 shows much higher levels of the metabolite. The assay for "bound" WR 1065, which is currently under development, is likely to be useful in determination of the fate of dosed ethiofos.

(7) Only low levels of ethiofos in plasma result after initial oral dosing of rhesus monkeys with ethiofos formulation.

(8) The rehesus monkey appears to be a more suitable animal model than the beagle dog for antiradiation drug dosing studies.

VI. RECOMMENDATIONS

(1) Continue to prepare additional oral dosage formulations of ethiofos to improve bioavailability of the drug.

(2) Continue in vitro evaluation of promising dosage forms of ethiofos by determining stability in acid solution and release rates in synthetic intestinal fluid.

(3) Continue to evaluate oral dosage forms of ethiofos in dosing experiments using the rhesus monkey as animal model.

(4) Continue determination of aging effects at 25°C and 37°C on the most promising oral dosage forms of ethiofos.

(5) Complete the development of a plasma assay for covalently bonded WR 1065 and show applicability to oral and IV dosing studies using rhesus monkey as animal model.

(6) Complete the development of a plasma assay for WR 33278, the disulfide metabolite of ethiofos. Show its applicability to IV dosing of ethiofos to beagle dog and to IV and oral dosing of ethiofos to the rhesus monkey animal models.

(7) Determine possible presence of mixed disulfides of WR 1065 and endogenous thiols in animal plasma following animal dosing with ethiofos or WR 1065.

(8) Determine distribution of ethiofos in animal model using ^{14}C-labelled ethiofos.

(9) Identify metabolites resulting from dosing of ethiofos in animal model.

(10) Complete hydrolysis rate study of WR 3689.

(11) Undertake additional ethiofos dosings with extended infusion times.

VII. LITERATURE CITED

1. L.M. Shaw, H. Bonner, A. Turrisi, A.L. Norfleet and D.J. Glover, J. Liq. Chromatogr., 7, 2447-2465 (1984).

2. D.J. Mangold, E.P. McGovern and P. Steele, "The Development of an HPLC Assay for 2-(3-Aminopropylamino)ethanethiol (WR 1065) in Plasma", Interim Report 4, February, 1984, Contract DAMD17-80-C-0128.

APPENDIX A

(Study Report 5)

AD _____

Study Report 5
Measurement of Ethiofos (WR 2721) in Plasma: Preliminary Pharmacokinetics in the Beagle

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January 1985

Support By
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland

Contract DAMD17-80-C-0128

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REPORT DOCUMENTATION PAGE

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1. REPORT NUMBER

2. GOVT ACCESSION NO.

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)

Measurement of Ethiofos (WR 2721) in Plasma: Preliminary Pharmacokinetics in the Beagle

5. TYPE OF REPORT & PERIOD COVERED

Study Report No. 5
9/1/84 - 10/16/84

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)

Nollie F. Swynnerton, Donald J. Mangold, Thomas M. Ludden

8. CONTRACT OR GRANT NUMBER(s)

DAMD17-80-C-0128

9. PERFORMING ORGANIZATION NAME AND ADDRESS

Southwest Research Institute
Post Office Drawer 28510, 6220 Culebra Road
San Antonio, Texas 78284

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

11. CONTROLLING OFFICE NAME AND ADDRESS

U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland 21701

12. REPORT DATE

January 1985

13. NUMBER OF PAGES

14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)

15. SECURITY CLASS. (of this report)

Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Distribution limited to U.S. government agencies only; contractor performance evaluation; September 1984. Other requests for this document must be referred to the Commander, U.S. Army Medical Research and Development Command (ATTN: SGRD-RMS), Fort Detrick, Frederick, Maryland 21701.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Antiradiation; radioprotector; WR 2721; ethiofos; WR 80855; plasma assay pharmacokinetics

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report contains a description of an analytical procedure for the determination of ethiofos in plasma and its application in preliminary pharmacokinetics in the beagle dog.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Ethiofos [S-2-(3-aminopropylamino)ethyl phosphorothioate, WR 2721)] is a radioprotective drug being investigated by the U.S. Army and human trials are being carried out by the National Cancer Institute. As part of a program with the U.S. Army Medical Research and Development Command, we have been determining plasma concentrations of this compound following IV administrations of beagle dogs.

We have previously reported a sensitive, precise analytical method for determination of the drug in plasma.[1,2,3] The method, using high performance liquid chromatography (HPLC) with fluorescence detection, was used in preliminary intravenous dosings of the drug in which a rapid rate of disappearance was observed over a thousand-fold concentration range (>1000 to <1 μg/mL). However, an apparent terminal phase with a long half-life was observed in these dosings with very low drug levels being measured for extended periods (up to 6 days). The possibility of low-level interferences generated by the drug dosing itself is always present in such experiments; therefore, a separation more selective for unchanged material was sought in these pilot investigations.

Investigation of the HPLC separation of ethiofos and its homolog, S-3-(4-aminobutylamino)propyl phosphorothioate (WR 80855) from endogenous plasma components has shown that dramatic changes in selectivity may be induced by changing the polar modifier used in the mobile phase. Development of a more selective HPLC system and its application are reported herein.

II. METHODS AND MATERIALS

A. Instrumentation

A Waters Associates Model 244 Liquid Chromatograph and a Laboratory Data Control (LDC) modular HPLC system were used. Both were fitted with a Rheodyne Model 7125 injector, an LDC Fluoromonitor III Fluorescence Detector and a Waters Associates RCM-100 Radial Compression Module with a 5 μ μBondapak C-18 cartridge. The mobile phase was a mixture of acetonitrile:ethanol:water (20:8:72), 0.01 M in dodecyltriethylammonium phosphate, pH of 2.8 and a flow of 2.0 mL/min. Injection volume was 50 μL. The analytical column was protected by a Whatman, Inc. guard column drypacked with Whatman, Inc. Pellicular ODS C-18. The detector excitation source was a 340-380 nm low pressure mercury and phosphor conversion lamp. A 370 nm bandpass filter and a 418-700 nm cutoff filter controlled excitation and emission wavelengths.

B. Reagents

USP absolute ethanol was purchased from U.S. Industrial Chemicals Company and HPLC-grade acetonitrile from J.T. Baker Chemical Company. Alkyltriethylammoniumphosphates were purchased from Regis Chemical Company as 0.5 M solutions in water. Fluorescamine was purchased from Aldrich Chemical Company and fresh solutions (5 mg/mL) were prepared weekly from reagent grade acetone which had been stored over 4A molecular sieves. The Walter Reed Army Institute of Research furnished ethiofos as the trihydrate (Lot AX, Bottle No. BK02762) in >99.0 percent purity and the internal standard WR 80855 as the dihydrate [Bottle No. ZP39150 (Experiment 10) and BK69918 (Experiments 14, 17 and 18)].

C. Sample Preparation

Plasma obtained by centrifugation of the whole blood samples was chilled in polyethylene vials, the internal standard was added (in 0.05 M sodium borate-potassium chloride pH 10 buffer solution) and the mixture was quick-frozen in a dry ice/isopropyl alcohol bath. The time from sample drawing to freezing was standardized and explicit. Typical sample volumes were 150 µL of plasma and 150 µL of internal standard. Samples were stored at -75°C until time of analysis. Immediately after thawing at room temperature the samples were treated with 200 µL of a 0.05 M sodium borate-potassium chloride buffer (pH 7.6) and, while the mixture was being agitated with a vortex mixer, 250 µL of the fluorescamine reagent was added. After mixing for about 60 seconds an additional 250 µL portion of reagent was added and agitation was continued 20-30 seconds. The resulting mixture was pressure-filtered through a 0.45 µm filter and the clear filtrate was injected onto the HPLC column.

D. Animal Dosing Experiments

The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards set forth by the "Guide for the Care and Use of Laboratory Animals," DHEW Publication NIH 74-23.

Beagle dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, Michigan. Two one-year old male beagle dogs weighing approximately 13 kg (quarantined 15 days to assure good health) had indwelling catheters placed into the cephalic vein (drug infusion) and

jugular vein (blood sampling). Catheters were placed aseptically under local anesthesia (2% xylocaine subcutaneous at the catheter injection site). The dogs were trained to quietly remain sitting on a table during the experimental period; however, ocassionally they were manually restrained. After an overnight fast the dogs received 150 mg/kg body weight of ethiofos during a 10-minute infusion. Ethiofos concentration in the 0.9% saline infusion solution was 100 mg/mL. The dogs remained calm during the experiment. All animals wretched and vomited during the infusion and postinfusion periods. The animals were returned to their kennels approximately 6 hours after dosing, their condition being unremarkable, and were allowed both food and water. We performed two experiments on each dog, allowing at least 35 days between each experiment.

Blood samples (3.0 mL) were withdrawn into EDTA vacutainers at the times indicated in Table 4 and immediately prepared as described in C above.

The data from each experiment were fitted to one-, two- and three-compartment open pharmacokinetic models with zero-order input of drug into the central compartment at a rate of 15 mg/kg/min for 10 minutes and first order elimination from the central compartment. Only data through 120 minutes were included because concentrations at later times were below the limit for accurate quantitation. The data were weighted by the reciprocal of the observed concentration. Kinetic parameters relatable to physiologic phenomena, i.e., volumes and clearances, were obtained iteratively using the digital computer program NONLIN.[4] An F-test[5] was used to assess statistical differences among the models.

III. RESULTS AND DISCUSSION

Investigation of a homologous series of alkyltriethylammonium phosphates (alkyl = pentyl, hexyl, heptyl, octyl and dodecyl) produced a mobile phase system which appeared to be highly selective for both unchanged drug and the homolog used as an internal standard. With dodecyltriethylammonium phosphate at a concentration of 0.01 M as the polar modifier in the mobile phase, drug and internal standard were separated from each other and from endogenous materials in beagle plasma in <30 minutes (Figure 1). Absence of interference was demonstrated by analysis of a plasma blank (Figure 2). With this system the lower limit of detectability was 0.05 µg ethiofos/mL plasma with precision (CV <10%) obtainable down to about 0.1 µg/mL. Endogenous materials were not separated from the drug and/or internal standard when the other substituted ammonium salts were used as the polar modifier.

The mobile phase is a variant of that used in the analysis of samples from earlier dosing studies. Formerly, tetrabutylammonium phosphate (TBAP) was used as the polar modifier of the acetonitrile:ethanol:water system. Although no interferences were detected during the development of the separation system which used TBAP, plasma samples taken from the beagles following infusion of ethiofos apparently contained low levels of the drug up to days after the administration. We now conclude that a small interference peak with the same elution time as ethiofos led to erroneously high values being calculated for the earlier dosings. Recent dosings using the new mobile phase in the plasma analysis do not exhibit a long terminal half-life and re-analysis of stored samples from the early experiments show lower (or zero) ethiofos concentations at extended postinfusion times.

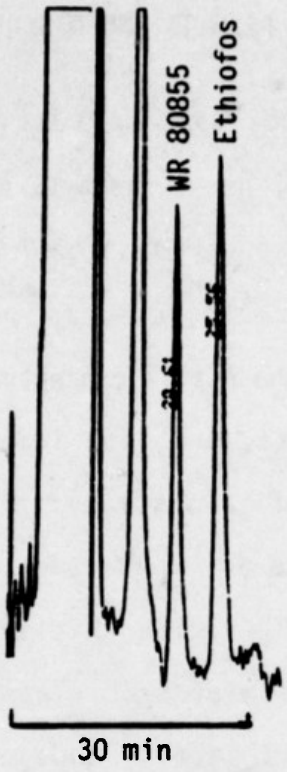

Figure 1. Chromatographic trace of beagle plasma spiked with 1.0 µg/mL of both ethiofos and WR 80855. Detector attenuation was 5X.

Figure 2. Chromatographic trace of beagle plasma blank. Detector attenuation was 5X.

It should be emphasized that the new procedure varies from the previous one only in the mobile phase. Elution times for drug and internal standard are not significantly changed although the order of elution is inverted. Sensitivity and linearity of the method, as judged by detector response and standard curves, are unchanged from the previous system. Rigorous characterization of the analytical method after change of the mobile was considered to be unnecessary. Appendix A contains the characterization of the method with the previous mobile phase. This was previously reported in Interim Report 3 prepared under this contract.

Concentration-time profiles for the dosing experiments are presented in Figures 3-6. Data sets for experiments 14, 17 and 18 were adequately described (F-test) by the two-compartment model (Table 1). However, the two-compartment fit to the data from experiment 10 did not result in a statistically better description of the concentration profile. In spite of this the two-compartment model provided a better visual description of the data (Figure 6). The parameters for the models of best fit for all four experiments are summarized in Table 2. Because of the ambiguity in models for experiment 10, parameters for both the one- and two-compartment models for this data set are given in Table 3.

Greater than 99.8% of the area under each concentration-time curve (AUC) was contained within the 0 to 120 minute time period assuming, of course, that a slow terminal phase does not exist at lower concentrations. Even if such a phase does exist (experiment 10, Table 4) more than 98% of the AUC would still have been contained in the data for the 0 to 120 minute period if the terminal half-life was 3-4 hours. Therefore, estimates of clearance would be virtually unchanged.

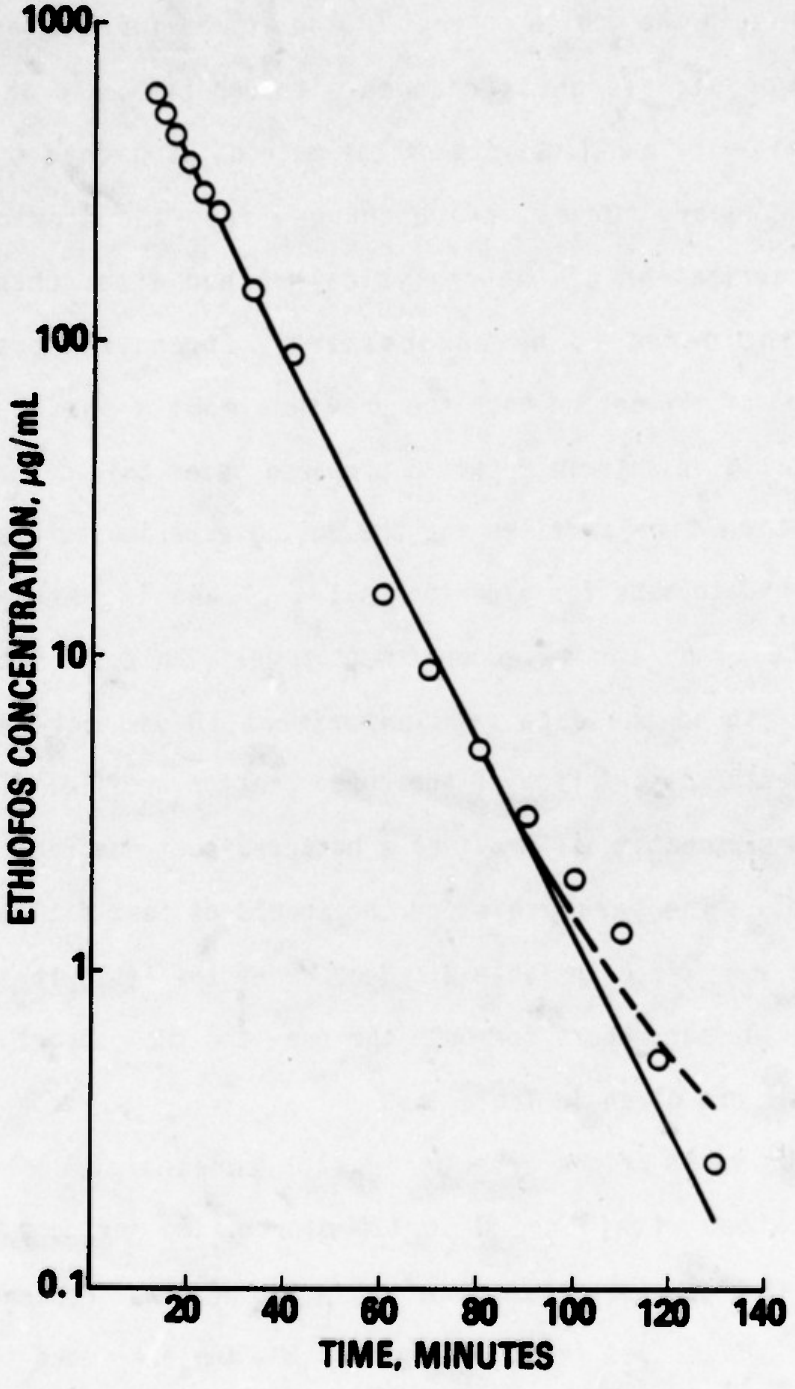

Figure 3. Ethiofos Pharmacokinetics--Experiment 10. The solid line is the concentration-time profile predicted by the one-compartment model. The dashed line is the portion of the concentration-time profile predicted by the two compartment model that differs from that predicted by the one compartment model.

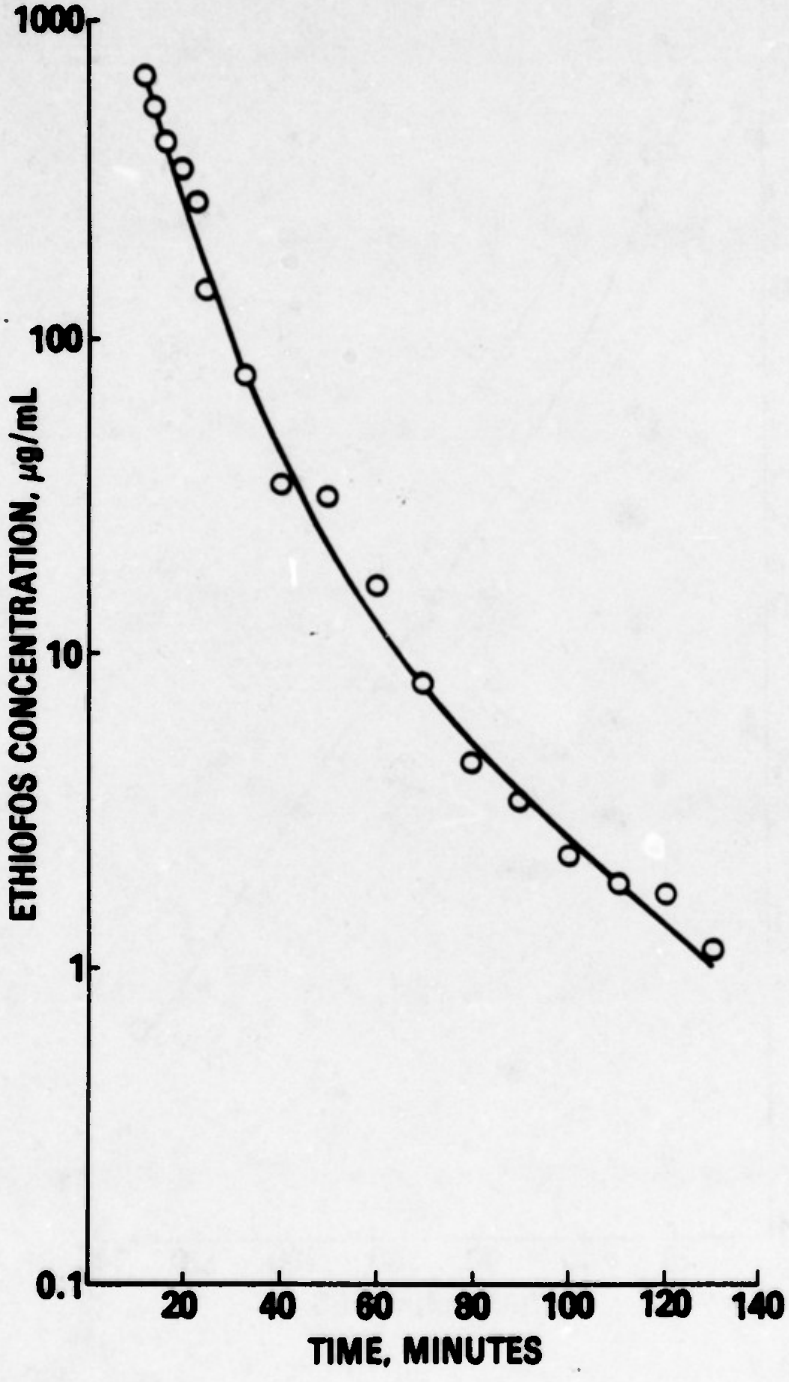

Figure 4. Ethiofos Pharmacokinetics--Experiment 14. The solid line is the concentration-time profile predicted by the two compartment model.

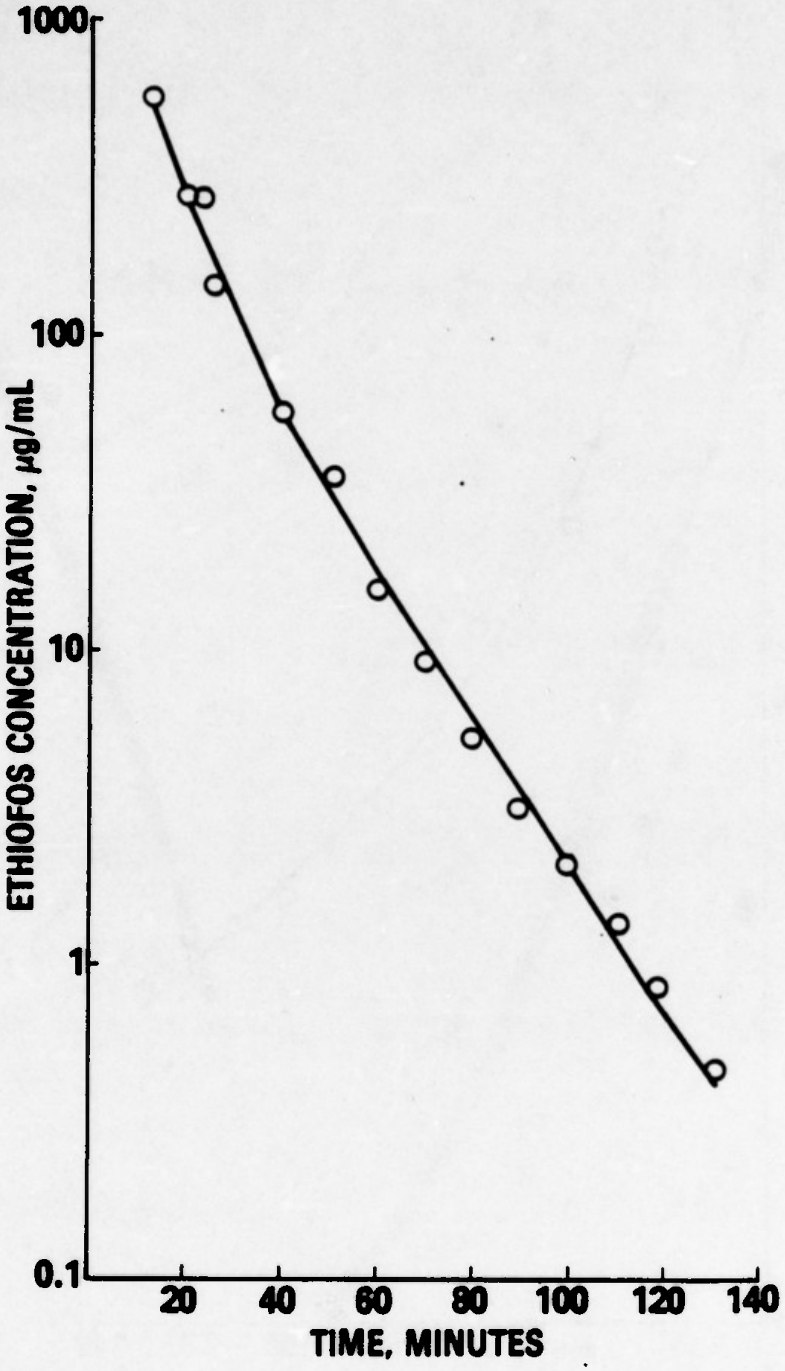

Figure 5. Ethiofos Pharmacokinetics--Experiment 17. The solid line is the concentration-time profile predicted by the two compartment model.

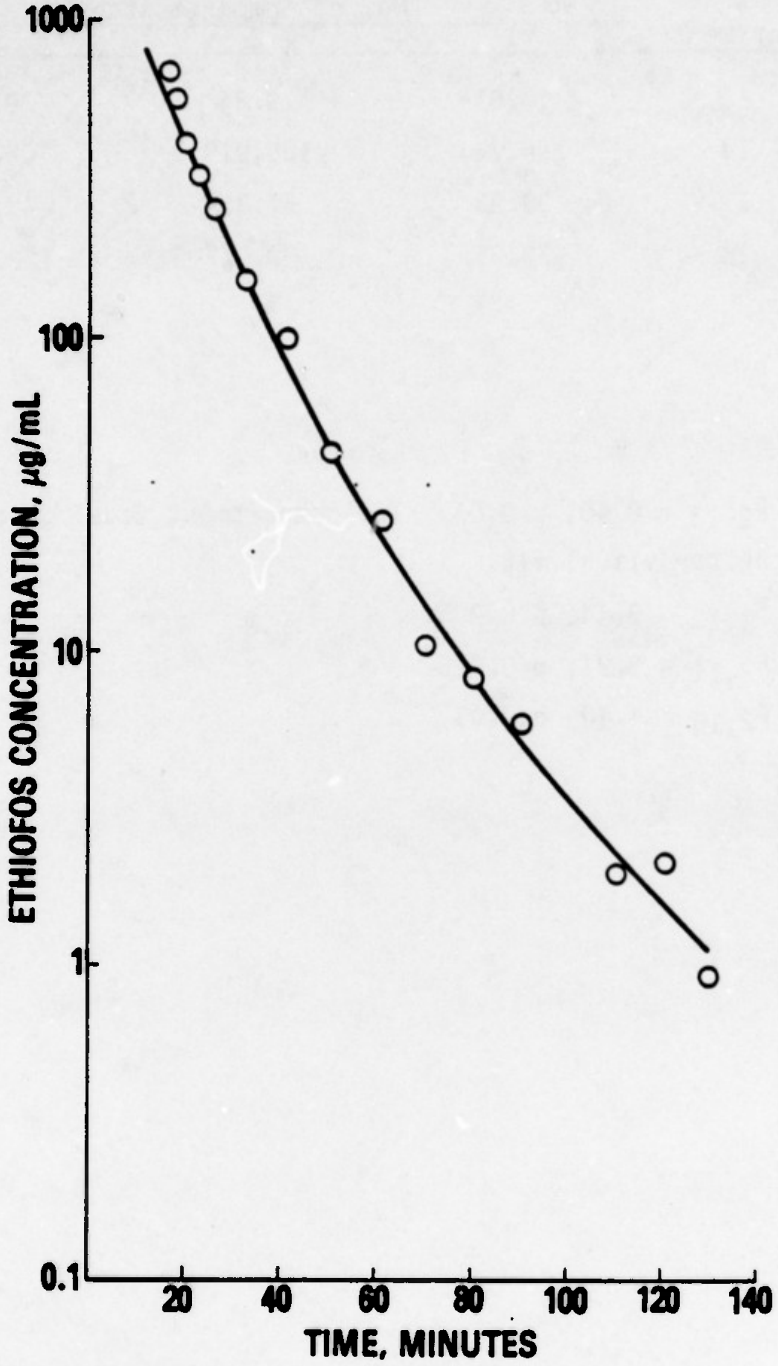

Figure 6. Ethiofos Pharmacokinetics--Experiment 18. The solid line is the concentration-time profile predicted by the two compartment model.

TABLE 1. WEIGHTED SUM OF SQUARED DEVIATIONS FOR FITTED DATA

Experiment	No. of Compartments 1	2	3
10	10.51[a]	9.84	9.79
14	259.74	105.91[b]	105.91
17	99.83	47.85[c]	47.88
18	172.96	103.17[d]	103.18

a. $F_{2,13} = 0.40$, $p>0.05$. Two-compartment model gave slightly better visual fit.

b. $F_{2,13} = 9.44$, $p<0.005$.

c. $F_{2,11} = 5.97$, $p<0.025$

d. $F_{2,13} = 4.40$, $p<0.05$

TABLE 2. ETHIOFOS PHARMACOKINETIC PARAMETERS

Parameter	Dog CSX-1		Dog BD-13	
	Experiment 10	Experiment 18	Experiment 14	Experiment 17
V_c, mL/kg	158	119	106	133
V_p, mL/kg	-	12.6	24.4	28.6
Cl_D, mL/min/kg	-	0.480	0.905	2.10
Cl_E, mL/min/kg	11.1	9.28	11.3	12.4
α, min^{-1}	-	0.0853	0.119	0.130
β, min^{-1}	0.0698	0.0348	0.0332	0.0528
$T_{1/2\alpha}$, min	-	8.12	5.82	5.33
$T_{1/2\beta}$, min	9.93	19.9	20.9	13.1

V_c - volume of central compartment

V_p - volume of peripheral compartment

Cl_D - distributional clearance

Cl_E - elimination clearance

α - distribution phase macro rate constant

β - elimination phase macro rate constant

$T_{1/2}$ - half life

TABLE 3. COMPARISON OF PARAMETERS FOR EXPERIMENT 10 FOR THE ONE- AND TWO-COMPARTMENT MODELS

Parameter	No. of Compartments	
	1	2
V_C, mL/kg	159	158
V_p, mL/kg	-	24.4
Cl_D, mL/min/kg	-	0.0770
Cl_E, mL/min/kg	11.1	11.1
α, min^{-1}	-	0.0701
β, min^{-1}	0.0698	0.00313

TABLE 4. PLASMA ETHIOFOS LEVELS FOLLOWING IV ADMINISTRATION OF BEAGLE DOGS

Post-infusion Time, Min	Dosing No. Plasma Levels of Ethiofos, µg/mL			
	10	14	17	18
2	599	664	567	680
4	504	537	-	687
6	438	417	-	563
9	362	338	276	420
12	291	268	276	307
15	256	144	146	250
22	143	76.3	98.1	151
30	90.6	34.2	58.2	98.2
40	41.0	31.4	36.1	42.5
50	15.7	16.2	15.9	25.5
60	9.11	7.92	9.45	10.5
70	5.11	4.57	5.42	8.06
80	3.12	3.42	3.25	5.79
90	1.97	2.28	2.11	3.93
100	1.32	1.87	1.33	2.00
110	0.53	1.71	0.83	2.07
120	0.25	1.18	0.46	0.93
180	ND	ND	0.15	0.07
240	ND	ND	0.15	ND
300	ND	-	0.21	ND
360	ND	-	0.11	ND
480	ND	-	0.09	-
720	ND	-	0.05	-
960	ND	-	0.03	-

ND = None detected
- = Samples not analyzed or no sample taken

The terminal half-life of ethiofos varied about two-fold among the four experiments and between the different days for each dog (Table 2). This variability in terminal half-life appears to be related primarily to interexperimental variations in distribution parameters, V_C, V_p and Cl_D. Elimination clearance, Cl_E, was similar among all experiments.

The kinetic parameters for ethiofos indicate that this compound has a relatively small volume of distribution, about 15% of body weight, with the largest portion of this volume being rapidly accessible to drug. The peripheral compartment is small and equilibrates slowly relative to drug elimination. The small volume of distribution and rapid clearance yield a very short terminal half-life of 10 to 20 minutes. Thus, ethiofos is rapidly eliminated with minimal reversible distribution. The high elimination clearance is compatible with biotransformation of ethiofos by hepatic and extrahepatic sites, including blood itself.

During the preparation of a second draft of this report results of a recent ethiofos dosing to a beagle became available. Total drug dose was 150 mg/kg as above, but an extended infusion time of 110 minutes was employed. This experiment is treated separately in Appendix B.

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APPENDIX A

CHARACTERIZATION OF HPLC ASSAY OF ETHIOFOS IN PLASMA

APPENDIX A
CHARACTERIZATION OF HPLC ASSAY OF ETHIOFOS IN PLASMA

A. Internal Standard

Among a number of candidates investigated as potential internal standards for the HPLC assay of WR 2721 (I), a homolog, S-3-(4-aminobutylamino)propyl phosphorothioate (WR 80855, II) was found to have essentially the same chemical behavior. Derivatization with fluorescamine was rapid and repeatable, and buffer solutions of WR 80855 and of WR 2721 had similar short-term stabilities.

Several chromatographic systems were able to separate WR 80855 from WR 2721 and from interfering constituents in beagle plasma. A mobile phase of acetonitrile/water (22:78) modified with 0.01 M tetrabutylammonium phosphate (TBAP) and a C-18 reverse phase column was satisfactory for most analyses but the system was unable to resolve a minor interference which coeluted with WR 2721 and corresponded to about 0.2-0.3 µg/mL of the drug. This interference could be separated using a mobile phase of acetonitrile/ethanol/water (16:7:77), 0.01 M in TBAP, but at the expense of a slightly longer chromatography time. Apparent sensitivity was also reduced by the resulting broader peaks.

B. Sensitivity

Replacement of the type of fluorescence detector used in previous studies resulted in a 10- to 20-fold increase in sensitivity. This detector also allowed determination of drug levels ranging from the minimum detectable limit of ~0.05 µg/mL to greater than 1000 µg/mL with no sample dilution or injection volume changes being required.

C. Linearity

Because of the wide range of drug levels expected to be encountered in pharmacokinetic studies, it was necessary to prepare several calibration curves, each covering a portion of the total range. This also facilitated the addition of varying amounts of the internal standard to correspond with the anticipated level of WR 2721. Four curves were constructed over the ranges 0-1.0, 0-10, 0-100, and 0-1000 µg WR 2721/mL plasma; internal standard levels were 2.22, 22.2, 222 and 1110 µg WR 80855/mL plasma, respectively. Linear regression of WR 2721/WR 80855 peak height ratios against WR 2721 concentration produced excellent fits with coefficients of determination $\geq$0.9986 (Table 1). Figures 1a-1d are plots of the four data sets. In general, use of integrated peak areas gave more scatter in the data than peak height measurements and occasionally gave anomolous values.

Standard curves constructed from the analysis of known amounts of WR 2721 in buffer solution were superimposable upon those obtained from analysis of the drug contained in plasma.

D. Accuracy and Precision

Plasma samples containing randomly-chosen levels of WR 2721 spanning the range of 0.25 to 770 µg/mL were assayed to determine the accuracy of the method. An average deviation of 5.5% from the actual value was obtained for eight determinations (Table 2).

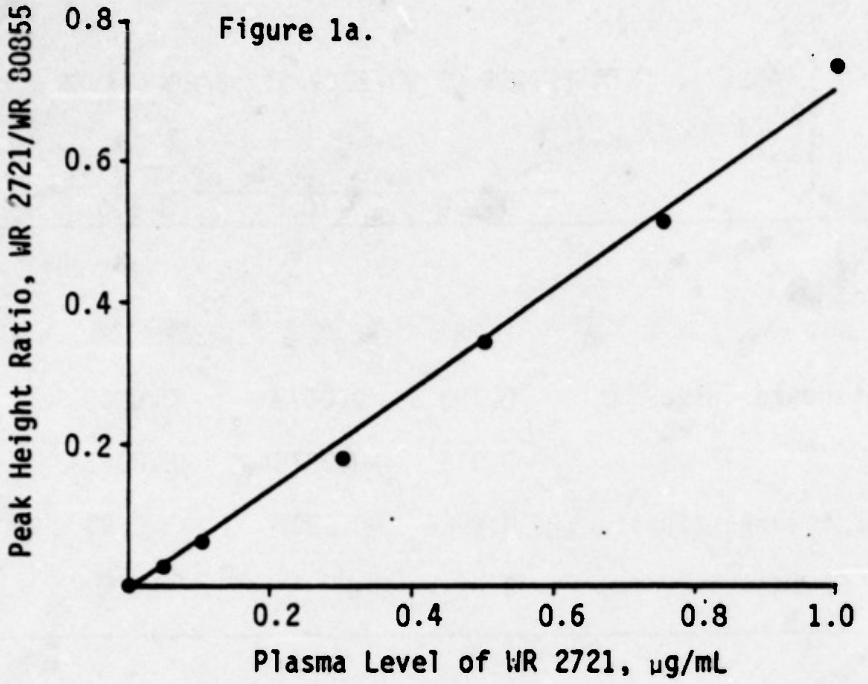
Precision estimates were obtained from replicate analyses of beagle plasma spiked with WR 2721 at the 0.5, 5, 50 and 500 µg/mL levels. The appropriate amount of WR 80855 was added as an internal standard. Coefficients of variation ranged from 1.4 to 5.0% withan average of 2.9% for the four levels (Table 3).

TABLE 1. STATISTICS OF WR 2721 STANDARD CURVES

	Plasma Levels of WR 2721, µg/mL			
	0-1.0	0-10.0	0-100	0-1000
Concentration of Internal Standard,[a] µg/mL	2.22	22.2	222	1110
Slope of Standard Curve[b]	0.722	0.0678	0.0102	0.00205
Intercept " "	-0.016	-0.00756	0.00428	0.00990
Coefficient of Determination	0.9986	0.9997	1.0000	0.9999
Degrees of Freedom	5	6	4	4

a. S-3-(4-aminobutylamino)propyl phosphorothioate, WR 80855.

b. Linear regression of (peak height of WR 2721)/(peak height of WR 80855) against concentration of WR 2721 in µg/mL.



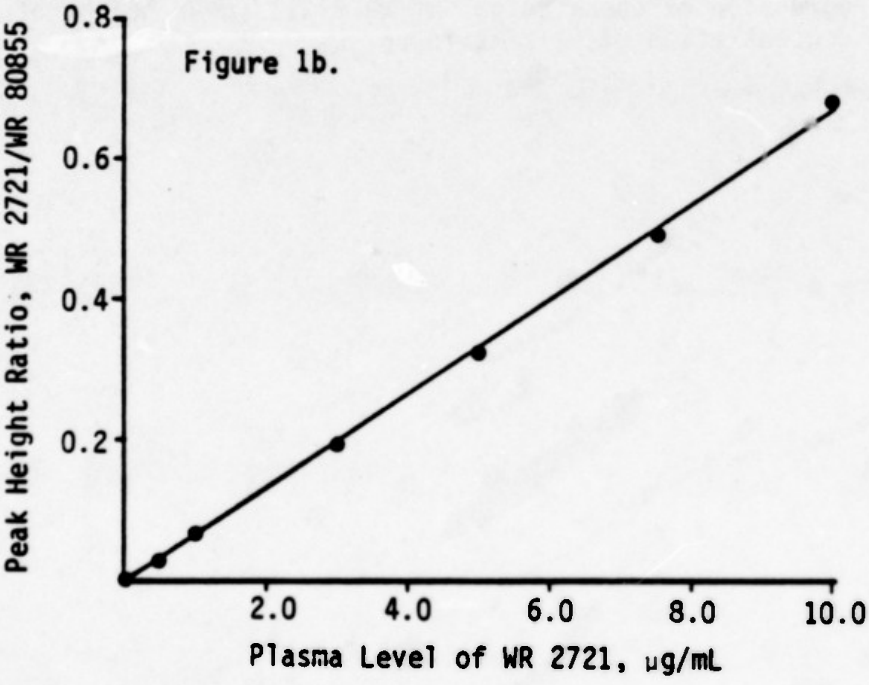
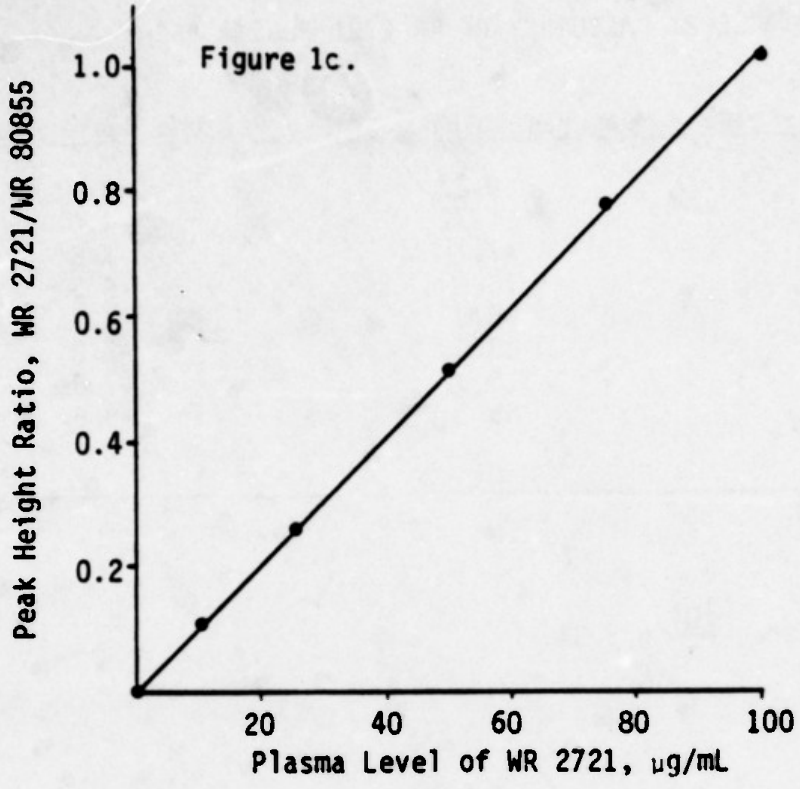


Figure 1a,b. Standard Curves. Samples of beagle plasma spiked with WR 2721 and WR 80855 as internal standard



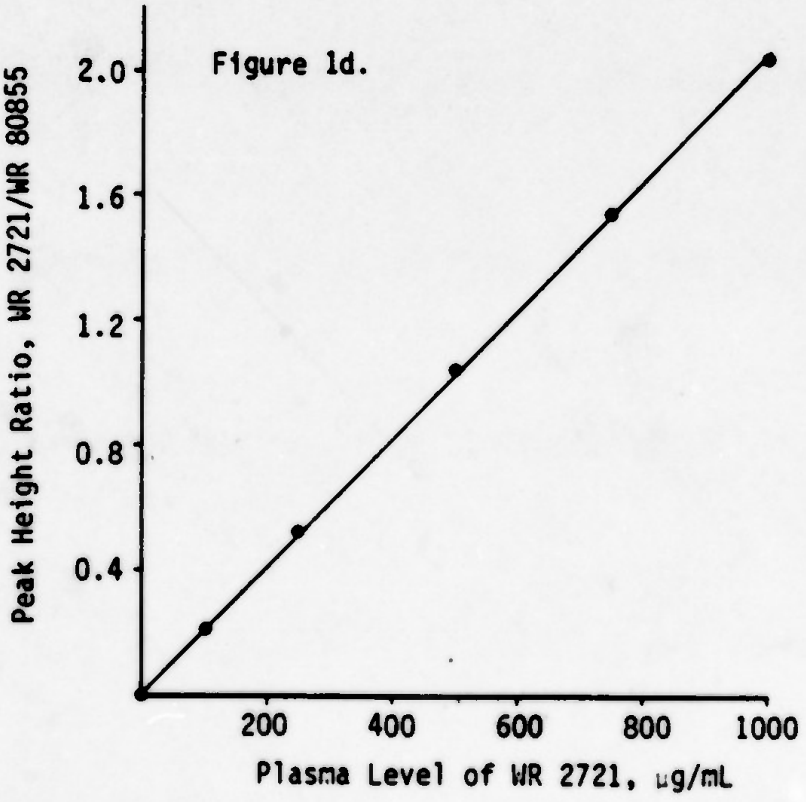


Figure 1c,d. Standard Curves. Samples of beagle plasma spiked with WR 2721 and WR 80855 as internal standard

TABLE 2. ACCURACY OF WR 2721 PLASMA ASSAY

Spike Level, µg/mL	Measured Level, µg/mL	Percent Deviation (D)
0.25	0.27	8.0
0.75	0.72	-4.0
8.0	7.7	-3.8
15.0	16.4	9.3
40.0	36.3	-9.2
90.0	83.0	-7.8
320	315	-1.6
770	765	-0.6

Average Deviation = $\frac{|D|}{n}$ = 5.5%

TABLE 3. PRECISION ESTIMATES OF WR 2721 ASSAY USING WR 80855 AS INTERNAL STANDARD[a]

WR 2721 Plasma Level, µg/mL	0.5	5.0	50	500
Number of Replicates	7	7	5	6
Average peak height ratio	0.576	0.258	0.516	1.06
Standard deviation	0.0206	0.0116	0.00734	0.018
CV, %	3.6	5.0	1.4	1.7

a. Mobile phase of CH_3CN/EtOH/water, 0.01 M in TBAP, 2.0 mL/min.

APPENDIX B

PHARMACOKINETIC MODELING OF DATA FROM LONG-TERM IV ADMINISTRATION OF ETHIOFOS TO A BEAGLE DOG

APPENDIX B
PRELIMINARY STUDY OF ETHIOFOS PHARMACOKINETICS IN THE BEAGLE USING A NEW HPLC ASSAY TO FOLLOW PLASMA CONCENTRATIONS

A. Protocol

1. Background

An HPLC assay for ethiofos has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

2. Objective

The objective of this pilot experiment is to collect plasma samples to assay for ethiofos following an intravenous dose of the drug.

3. Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 9:00 a.m. Dog will be fasting on day of experiment. Food will be withheld during the six hours of the study but the animal will have access to water throughout the experiment (Note 6).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited (Note 4).

IV infusion of the drug will be over a two hour period. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of ethiofos (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved to give a final concentration of 100 mg/mL ethiofos or 125 mg/mL ethiofos·$3H_2O$.

A sample of blood (10 mL) will be taken prior to infusion for standards and 3 mL taken immediately prior to the beginning of the infusion.

Blood samples (3 mL) will be collected during the infusion at the following time periods: 0, 30, 60, 90, and 120 min. A sample of the infusion solution will be taken for assay prior to start of the infusion and at the end. Post infusion blood samples (3 mL) will be collected at 2,

4, 6, 9, 12, 15, 22, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 180, 240, 300, and 360. The end of the infusion period (120 minutes) is set as time zero. The blood samples will be collected, immediately centrifuged, divided into aliquots, spiked with internal standards and stored frozen at -78°C prior to analysis (Note 7). The blood in the catheters will be replaced with heparinized saline (Note 8). The catheters will be removed at the end of the experiment.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Total formulation prepared will be 3.750 g of ethiofos as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Exact dose will be based on weight of dog.

2. Formulation of dose will be within one hour prior to infusion time.

3. Dose of 150 mg/kg should produce a maximum blood level of ethiofos of approximately 1500 ug/mL assuming total blood volume of dog is 1.5 L.

4. A tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

5. Ethiofos dose will be prepared within one hour of dosing. The drug may be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube day of test and the ethiofos dissolved by agitation. Sample for injection can be removed from tube by syringe.

6. Dog will be fed once/day in the afternoon after the blood samples are drawn to better accommodate dosing study. Dog should be on regular diet.

7. Detailed written procedures will be prepared for the preparation of the plasma samples.

8. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

9. Dog temperature during study should be measured rectally and recorded. Dog should be kept covered and on wooden base to maintain body temperature.

10. Ten mL sample will be centrifuged and plasma frozen for delivery to Joe Niño.

B. Log

DOG DOSING STUDY NO. 28 DATE: 8/16/84

Dog Breed: Beagle 1 Dog Weight 15.9 kg (35 lbs) Protocol Date: 8/8/84

Dose Ethiofos: 23.9 mL (WR 2721; 100 mg/mL anhydrous basis); 150 mg/kg

TIME	START	FINISH	RECTAL TEMPERATURE	REMARKS
Time	(min)	(min)		
Dosing Time				
I 0	9:32	9:32	103°F	Infusion started 9:33 AM
I 30	30:02	30:20		Resting
I 60	60:00	60:20		
I 93	93:00	93:30		Catheter out; use left cephalic vein for drawing samples
I 110	110:00	110:10		
P 2	2:00	2:53		
P 5	5:00	5:20		
P 7	7:60	7:81		
P 9			Not taken	Skipped-difficulty in draw
P 12	12:00	12:16		Used catheter for drawing sample in right leg
P 15	15:00	15:20		"
P 22	22:00	22:15	100°F	"
P 30	30:00	30:25		"
P 40	40:00	40:20	99.5°F	Resting
P 50	50:00	50:20		Resting
P 60	60:00	60:50		Resting
P 70	70:00	70:16		Resting
P 80	80:00	80:20		Resting
P 90	90:00	90:32	100.0°F	Resting
P 100	100:00	100:36		Resting
P 110	110:00	110:27	100.0°F	Resting
P 120	120:00	120:27		Resting
P 180	180:00	180:18	100.0°F	Resting
P 240	240:00	240:18		Resting
P 300	300:00	300:22	100.0°F	Resting
P 360	360:19	360:52		Unremarkable; returned to kennel 1720 hrs

NOTES: Dog checked for well being at 1900 hrs - condition good
10:00 3-mL control taken 9:15 AM
18:90 Licking
19:60 Wretching - little volume - vomiting <1 cc
27:40 Wretching - vomiting - approximately 5 cc
27:90 Vomiting
33:89 Wretching; Vomiting approximately 3 cc
42:38 Wretching approximately 1 cc
54:10 Wretching; little vomit
108:90 Wretching; dr heaves
109:50 Wretching
110:00 Finish infusion
P = 190 given 1 cup water - vomited at P = 194

C. Pharmacokinetic Modeling

1. Methods

The data from the experiment were fitted to a one-, a two- and a three-compartment open pharmacokinetic model with zero-order input of drug into the central compartment at a rate of 1.36 mg/min for 110 min and first-order elimination from the central compartment. The data were weighted by the reciprocal of the observed concentration. Kinetic parameters relatable to physiologic phenomena, i.e., volumes and clearances, were obtained iteratively using the digital computer program NONLIN[1]. An F-test[2] and the Akaike Information Criterion[3] (AIC) were used to assess statistical differences between the models.

2. Results

Concentration-time data are presented in Table Ib and the corresponding profile for the experiment is presented in Figure 1b. The parameters of the models are summarized in Table IIb. The data set for this experiment was described better by the two-compartment model than by the one-compartment model. The three-compartment model resulted in a better visual fit but was not statistically better. Several of the parameters for the three compartment model, including clearance, had large relative standard deviations.

2. Discussion

The most notable differences between the results of this long infusion experiment and the short infusion experiments are the higher clearance observed after long infusion and the evidence for a terminal elimination phase with a long half-life. However, there were insufficient data to adequately describe this longer phase. The higher clearance could be a result of incomplete administration of the dose. If the drug was unstable in the administered solution then the calculated clearance would be an over-estimate of the true clearance.

TABLE Ib. PLASMA ETHIOFOS CONCENTRATIONS FOLLOWING IV ADMINISTRATION OF 2.39 G OVER 110 MIN

Time, min	Ethiofos Conc., µg/mL	Comments
0	-	Infusion begun
30	56.2	
60	92.3	
90	81.0	
110	63.7	Infusion ended
112	75.6	
114	48.6	
116	35.1	
122	21.6	
125	17.3	
132	11.8	
140	6.8	
150	7.4	
160	7.8	
170	4.5	
180	2.9	
190	1.9	
200	1.7	
210	0.87	
220	0.56	
290	0.23	
350	0.16	
410	0.16	
470	0.06	Dog returned to kennel. Condition unremarkable.

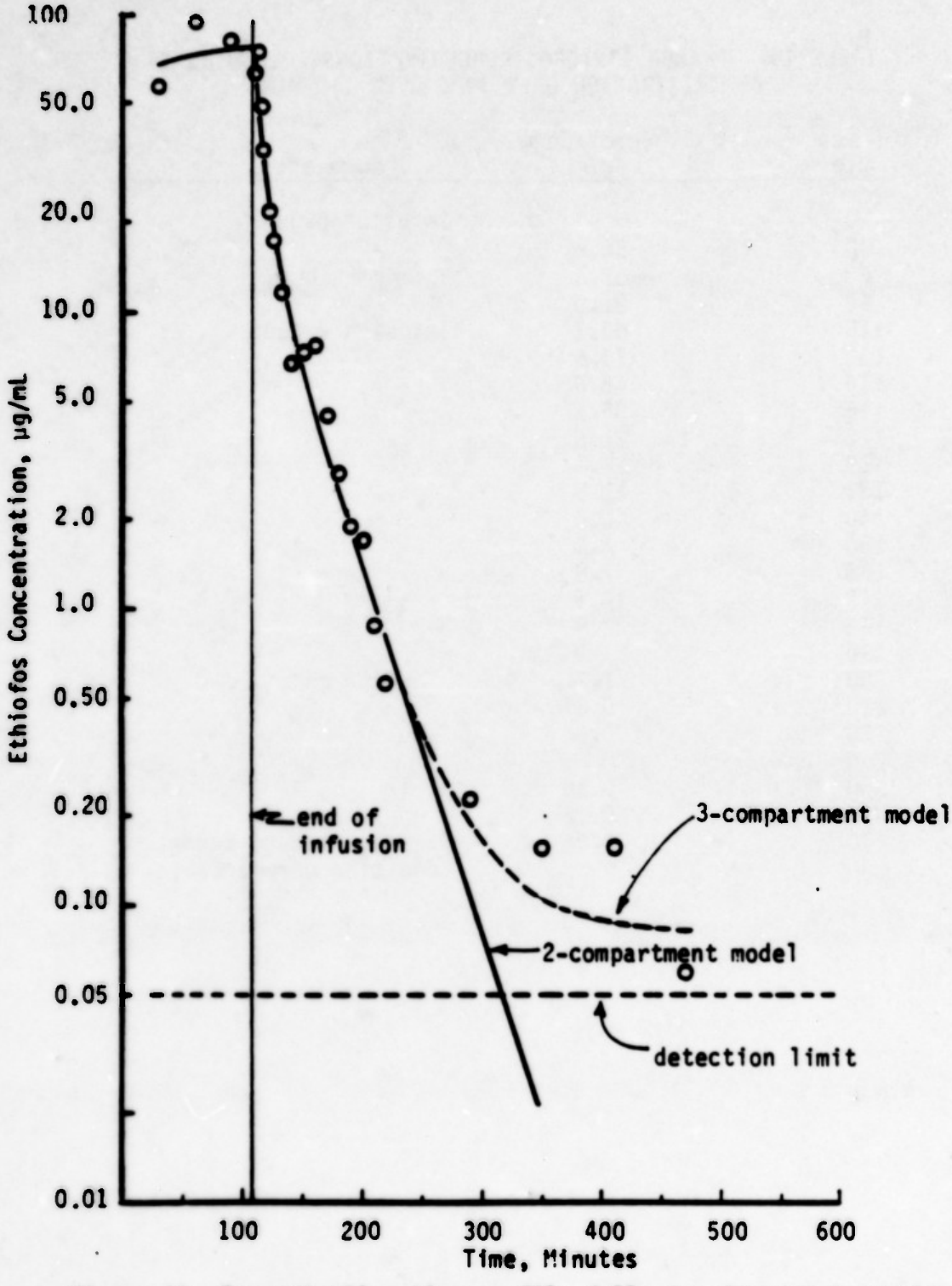


Figure 1b. Concentration-time profile following IV administration of ethiofos to a beagle dog. Dose of 150 mg/kg, infusion time of 110 minutes.

TABLE IIb. ETHIOFOS PHARMACOKIENTIC PARAMETERS

Compartments	1	2	3
V_C, L/kg	0.242(0.0358)	0.142(0.0308)	0.141(0.0333)
V_p1, L/kg	-	0.107(0.0228)	0.104(0.0319)
V_p2, L/kg	-	-	0.674(70.1)
Cl_D1, L/kg/min	-	0.00385(0.00166)	0.00394(0.00192)
Cl_D2, L/kg/min	-	-	0.00038(0.0189)
Cl_E, L/kg/min	0.0192(0.00125)	0.0177(0.00085)	0.0173(0.0182)
α, min	0.0793	0.160	0.162
β, min	-	0.0281	0.0293
γ, min	-	-	0.000552
$T_{1/2}$, α	8.74	4.33	4.28
$T_{1/2}$, β	-	24.7	23.7
$T_{1/2}$, γ	-	-	1255.4

V_C - volume of central compartment

V_p - volume of peripheral compartment

Cl_D - distribution clearance

Cl_E - elimination clearance

α - primary distribution phase macro rate constant

β - secondary distribution phase macro rate constant

γ - elimination phase macro rate constant

$T_{1/2}$ - half life

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APPENDIX B

(Interim Report 4)

AD _____

Interim Report 4
The Development of an HPLC Assay for 2-(3-Aminopropylamino)ethanethiol (WR 1065) in Plasma

Donald J. Mangold, Ph.D., Principal Investigator
Edward P. McGovern, Ph.D., Senior Research Scientist
Patricia Steele, Research Scientist

February 1984

Support By
U.S. Army Medical Research and Development Command
Fort Detrick, Frederick, Maryland

Contract DAMD17-80-C-0128

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1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
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		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Donald J. Mangold, Edward P. McGovern, Patricia Steele		8. CONTRACT OR GRANT NUMBER(s) DAMD17-80-C-0128
9. PERFORMING ORGANIZATION NAME AND ADDRESS Southwest Research Institute Post Office Drawer 28510, 6220 Culebra Road San Antonio, Texas 78284		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS U. S. Army Medical Research and Development Command Fort Detrick, Frederick, Maryland 21701		12. REPORT DATE February 1984
		13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Antiradiation compounds; WR 1065; plasma assay.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report contains a description of an analytical procedure for the determination of WR 1065 in plasma.

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SUMMARY

An HPLC plasma assay has been developed for the detection and quantification of the drug 2-(3-aminopropylamino)ethanethiol (WR 1065). The analyte is separated using a Whatman 5μ ODS-3 column with a mobile phase composed of acetonitrile/0.1 molar monochloroacetic acid pH 3.0 4:97, (v/v) containing 0.005 moles/L of dl-10-camphorsulfonic acid at a flow rate of 2.0 mL/min. 2-(5-Aminopentylamino)ethanethiol (WR 1729) is used as an internal standard. The detector, a Bioanalytical Systems, Inc. amperometric controller with an electrochemical transducer cell equipped with a single mercury/gold (Hg/Au) working electrode, assures both analyte specificity and sensitivity. The electrochemical transducer cell is operated at +0.15 volts (relative to silver/silver chloride) and is specific for thiols. The present detection limit is <0.05 μg (WR 1065)/mL plasma. The absolute retention times for WR 1065 and WR 1729 are 9 and 12 minutes, respectively. The assay uses 100 μL of plasma and requires a total chromatography cycle time of 40 minutes. The method has been found suitable for the detection of WR 1065 in plasma from a beagle dog after IV administration of either S-2-(3-aminopropylamino)ethyl phosphorothioate (ethiofos, WR 2721) or WR 1065. No interferences due to plasma constituents or drug metabolites were observed.

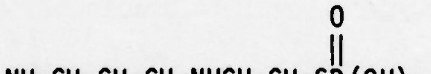
TABLE OF CONTENTS

LIST OF TABLES

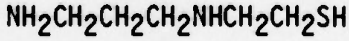
LIST OF FIGURES

I. INTRODUCTION

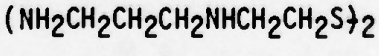
Since 1959 the U. S. Army Medical Research and Development Command, Walter Reed Army Institute of Research, has synthesized and evaluated many compounds for possible use in protecting personnel from the harmful effects of ionizing radiation. One of the compounds, S-2-(3-aminopropylamino)ethyl phosphorothioate, (ethiofos or WR 2721), 1, appears to be the most promising.[1] It is believed that in the body the drug is transported intact into the tissue where it is enzymatically cleaved to yield 2-(3-aminopropylamino)ethanethiol, 2, (WR 1065).[2-4] Free radicals produced by radiation are then scavenged by reaction with the sulfhydryl (SH) group of 2.[5] Oxidation of WR 1065 can yield the disulfide 3 which is a metabolite of concern.



Ethiofos, WR 2721 1



WR 1065 2



WR 33278 3

WR 1729 4

In order to elucidate the pharmacokinetics of ethiofos and WR 1065, a sensitive and reliable plasma assay procedure was required. (Methods for the assay of ethiofos have been the subject of earlier reports on this project.) Although several methods for quantitating WR 1065 have been reported,[2,4,6,7] no single procedure allows rapid, sensitive and selective determination at the microgram and submicrogram per milliliter plasma levels.

Recent publications[8-10] describing the successful determination of thiol containing compounds by high performance liquid chromatography (HPLC) with electrochemical detection (ECD) suggested this approach would have merit for WR 1065 determinations. In May of 1983 research commenced on the feasibility of using HPLC/ECD as the basis for an analytical method to determine WR 1065 at the µg/mL level in beagle plasma. In November of 1983 the results obtained were presented at the Chemical Modifiers in Cancer Treatment Symposium held in Banff, Alberta, Canada. The salient achievements at that time were:

- analyte separation by reverse phase HPLC using isocratic conditions;
- suitability and use of WR 1729 [$H_2N(CH_2)_5NH(CH_2)_2SH$] as an internal standard;
- no observed chromatographic interferences:
- detection using an electrochemical cell with Hg/Au electrodes providing both selectivity and sensitivity;
- quantification at the 1 µg/mL WR 1065 level in plasma;
- rapid disappearance of WR 1065 from beagle plasma after dosing with ethiofos.

As a result of discussions held during and subsequent to the conference in Banff, the following primary objectives were selected for immediate investigation:

- determination of the stability of WR 1065 in plasma, buffers and preserved plasma;
- improvement of the sensitivity to permit quantification at the 0.05 µg/mL plasma level;
- determination of the stability of ethiofos (WR 2721) in preserved plasma;
- assay of plasma samples from ethiofos and WR 1065 IV infusions.

II. MATERIALS AND METHODS

A. Instrumentation

Either an IBM Instruments, Inc. Model LC/9533 ternary gradient liquid chromatograph or an LDC Constametric III Liquid Chromatograph was used with a Bioanalytical Systems, Inc. (BAS) electrochemical detector. The detector was composed of a model LC-4B amperometric controller equipped with a model TL-6A transducer cell and a Hg/Au working electrode. The electrochemical cell was operated at a working potential of +0.15 V versus silver/silver chloride. Samples were injected using a Rheodyne Model 7125 injector with either a 20-µL or 100-µL sampling loop. Separations were carried out on a Whatman 250 mm x 4.6 mm ID Partisil 5 ODS-3 or a BAS 250 mm x 4.6 mm ID Biophase ODS 5µ prepacked column. The analytical column was protected from strongly adsorbed analytes by a Bioanalytical Systems, Inc. guard column containing a replaceable 30 mm x 4.6 mm cartridge packed with a 5 µm spherical C-18 packing. The mobile phase was either acetonitrile/0.1 M chloroacetic acid, pH 3.0, 3:97 or 4:96 modified to be 0.005 M in dl-10-camphorsulfonic acid (CSA) at a flow rate of 2.0 mL/min. If after 5 or 6 sample injections small changes in analyte resolution were observed, the mobile phase was changed via a linear gradient over 15 minutes to acetonitrile/0.1 M monochloroacetic acid 90:10, to flush the column, otherwise analyses continued until resolution deteriorated. In any case the column was flushed at the conclusion of the day's activities. Re-equilibration with the analytical mobile phase was achieved with a linear gradient prior to continuation of analyses. The condition of the Hg/Au electrode was evaluated by daily standard injections. If inadequate

detector sensitivity or erratic detector response was noticed, a freshly coated Hg/Au cell was introduced and allowed to equilibrate overnight before further analyses were performed.

B. Reagents

Acetonitrile and methanol were Baker analyzed HPLC reagent grade. Chloroacetic acid (99%) and dl-10-camphorsulfonic acid (98%) were purchased from Aldrich Chemical Company and used without further purification. Water for HPLC use was obtained from a Milli-Q® Water Purification System. All other chemicals were reagent grade quality. WR 1065, bottle number (BN) BK 05030, WR 2823 [S-2-(5-aminopentylamino)ethyl phosphorothioate], Lot AV, BN 25575 and WR 2721 trihydrate, BN BK 02762 were furnished by Walter Reed Army Institute of Research. WR 1729, [2-(5-aminopentylamino)ethanethiol] was prepared from WR 2823 by acid hydrolysis following the method of Tabachnik et al.[6] for the conversion of WR 2721 [S-2-(3-aminopropylamino)-ethyl phosphorothioate] to WR 1065.

Standards containing WR 1065 and WR 1729 were prepared by dissolution of the compounds in 0.1 M monochloroacetic acid/0.2 M perchloric acid 1:3. Standards containing WR 2721 were prepared by dissolution in 0.05 M sodium borate/potassium chloride pH 10 buffer.

C. Sample Preparation

Plasma (100 µL) and 400 µL of internal standard solution (WR 1729) were placed in a 1.0 mL polyethylene centrifuge tube, mixed and analyzed immediately. After mixing, the tube was centrifuged for 1.0 minute (Eppendorf Model 5414 Micro Centrifuge) to separate precipitated protein and a portion (20 or 100 µL) of the supernatant was injected onto the HPLC column.

Alternatively 100 µL of plasma was mixed with 400 µL of internal standard solution, quickly frozen in a dry ice/isopropyl alcohol bath and stored frozen at -78° until the time of analysis. Each frozen sample was then individually thawed at room temperature and treated as described above.

D. Care of Electrochemical Cell

During the past several months, much has been learned about the idiosyncrasies of this sensitive detector. The detector is extremely sensitive to external electromagnetic fields and must be placed inside a Faraday cage. The chromatographic systems must be passivated with 6N nitric acid followed by copious rinsing with either phosphate buffer (pH 7.5) or water. The pump heads must produce a pulseless flow or intolerable baseline noise will result. Internal standards and injection standards are necessary as the sensitivity of the cell may change during an 8 hour period. This is unavoidable as the cell surface is a reactant and slowly erodes with time and must be resurfaced (repolished and amalgamated with mercury). Extreme care must be taken to keep glassware clean and free of metal ions and other contamination. Very small currents are measured (<1nA in some cases) and trace impurities may result in discarding an entire batch of mobile phase.

No problems yet encountered have proven insurmountable, although some have been frustrating. The detector is well suited for specialized analyses; it is rugged and requires only appropriate maintenance to insure its efficiency.

E. Animal Dosing Experiments

The laboratory animal facilities and animal care program of Southwest Research Institute have been reviewed and accredited by the American Association for Accreditation of Laboratory Animal Care according to standards as set forth by the "Guide for the Care and Use of Laboratory Animals" DHEW Publication, NIH 74-23.

Healthy male beagle dogs were purchased from Laboratory Research Enterprises, Inc., Kalamazoo, Michigan, and used in pilot dosing experiments to test the analytical method. Two dogs were dosed on 3 occasions; ethiofos was dosed in two experiments and WR 1065 in another. Details of each study are presented in Table I. The dogs were dosed intravenously in the cephalic vein with a 0.9% saline solution containing each drug. Blood samples (3 mL) were withdrawn into an EDTA Vacutainer® from a cannula placed in the jugular vein. In Dosing Studies No. 8 and 9, each blood sample was immediately chilled in an ice-water bath and then centrifuged (app. 10 minutes). A 100-µL aliquot of the separated plasma was added to 400 µL of the internal standard solution (WR 1729:10 µg/mL), the mixture was agitated using a Vortex mixer and then quick-frozen in a dry ice/isopropyl alcohol bath. In Dosing Study No. 10, each blood sample was immediately centrifuged for about one minute using a microcentrifuge. A 100 µL aliquot of the separated plasma was added to 400 µL of the internal standard solution (WR 1729:10 µg/mL), the mixture was agitated using a Vortex mixer and then quickly frozen in a dry ice/ isopropyl alcohol bath. Samples were stored at -78° until time of analysis when they were thawed at room temperature and immediately treated as described in the Sample Preparation Section.

TABLE I. ANIMAL DOSING STUDIES

	Animal Dosing Studies		
	No. 8	No. 9	No. 10
Date	12/14/83	1/11/84	1/25/84
Dosing (IV)	Ethiofos	WR 1065	Ethiofos
Bottle No.	BK02762	BK05030	BK02762
Beagle No.	CSX-1 (No. 1)	BD-13 (No. 2)	CSX-1 (No. 1)
Dog weight (kg)	15.69	12.96	14.54
Dose (mg/kg)	150	60	150
Infusion period (min)	8.8	10	10

III. RESULTS AND DISCUSSION

A. Internal Standard

Several candidate internal standard compounds were screened for their applicability to this analysis; however, only two, WR 1729 and WR 251833 (2-(4-aminobutylamino)propanethiol), had appropriate retention characteristics. WR 1729 was chosen, as its absolute retention time of 12 minutes was nearest to that for WR 1065 which was 9 minutes. WR 251833 did not elute until 19.4 minutes. The mobile phase composition was acetonitrile/0.1 M monochloroacetic acid 3:97. WR 1065 and WR 1729 under the assay conditions are 100% resolved.

B. Linearity of Detector Response, Assay Precision and Accuracy

For these data a 20 µL injection volume and mobile phase of acetonitrile/0.1 M monochloroacetic acid (3.97) modified to be 0.005 M in dl-10-camphor sulfonic acid was employed. The linearity of the detector response over the assay range (1-500 µg/mL) was investigated and three separate calibration curves were constructed over the expected ranges of WR 1065 concentrations. Table II presents the statistics for the three standard curves, while Figure 1 shows representative chromatograms obtained over the calibration range.

The method precision was calculated by the analysis of replicate fortified plasma samples which give a coefficient of variation (CV) of less than 4% (Table III) from 5 to 500 µg/mL WR 1065.

The statistics describing the behavior of the three calibration curves (Table II) show the linearity of the peak height ratio versus concentration function within each of the defined ranges ($R^2 > 0.97$). It is apparent,

TABLE II. STATISTICS OF WR 1065 STANDARD CURVES

	Plasma Levels of WR 1065, µg/mL		
	1-10	10-100	100-500
Concentration of Internal Standard, µg/mL*	7.5	75	750
Slope of Standard Curve**	0.26	0.024	0.0016
Intercept of Standard Curve	-0.084	0.19	0.42
Coefficient of Determination	0.9945	0.9950	0.9737
Degrees of Freedom	3	3	2

*WR 1729, 2-(5-aminopentylamino)ethanethiol.
**Linear regression of (peak height of WR 1065)/(peak height of WR 1729) against concentration of WR 1065, µg/mL.

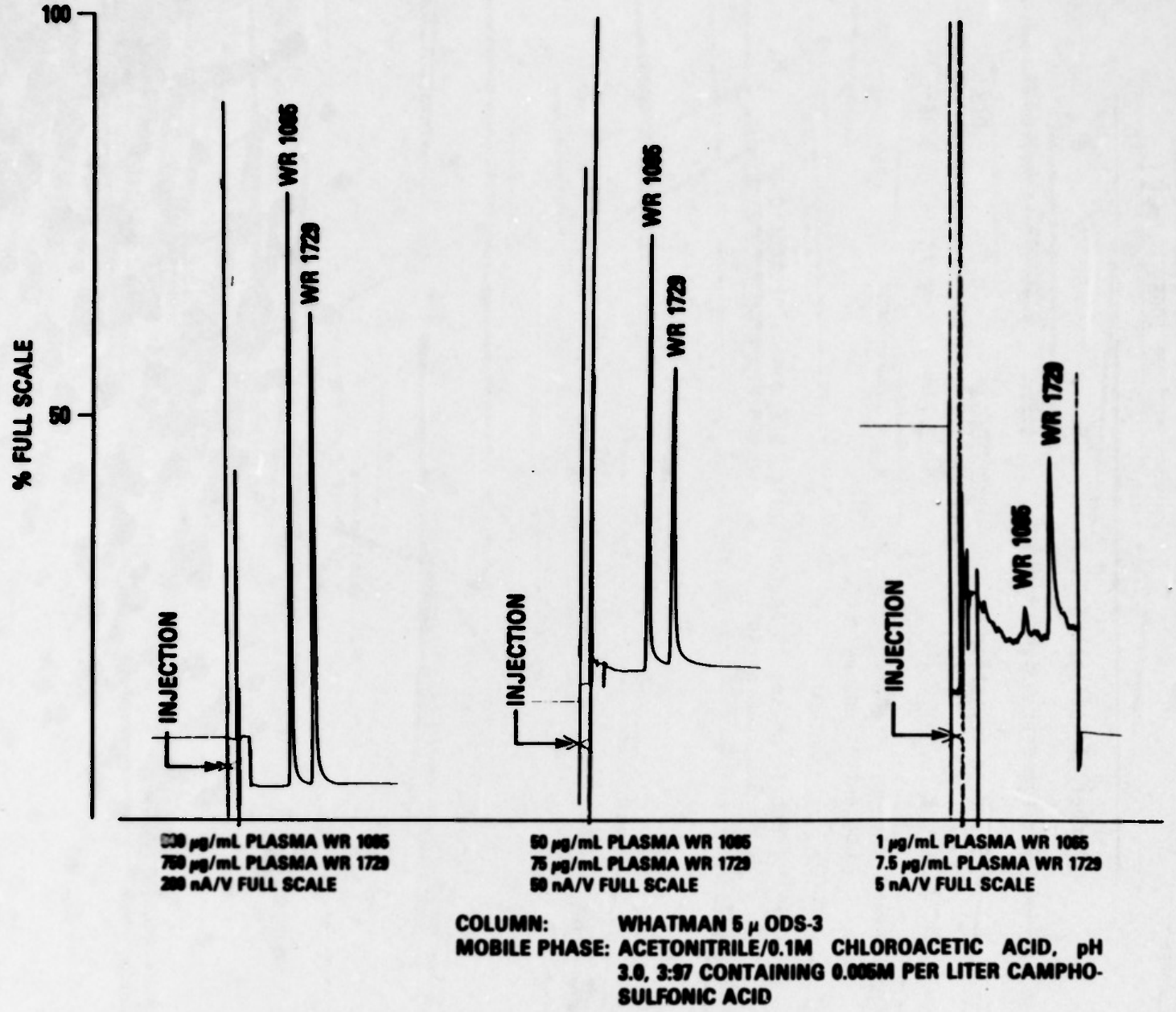


Figure 1. Chromatography of WR 1065 and WR 1729 Over the Range of 500 to 1 µg/mL of Plasma

TABLE III. PRECISION ESTIMATES FOR WR 1065 ASSAY USING WR 1729 AS AN INTERNAL STANDARD

	Plasma Levels of WR 1065, µg/mL		
	5.0	50	500
Number of Replicates	5	5	5
Average Peak Height Ratio	1.35	1.402	1.25
Standard Deviation	0.037	0.039	0.048
CV, %	2.74	2.78	3.86
Internal Standard Concentration, µg/mL	7.5	75	750

**Mobile phase: 3% Acetonitrile, 97% monochloroacetic acid 0.1 M, pH 3.0, camphorsulfonic acid 0.005 M. Flow rate 2 mL/min.

however, from the slope and intercept differences between the three linear regions that the overall curve shape is not linear but tends to flatten slightly at the higher concentration levels. The accuracy, as determined from the analysis of fortified plasma samples, was satisfactory. An average absolute deviation of 6.1% was obtained (Table IV).

C. Stability of Samples

1. WR 1065

Initial WR 1065 stability investigations (Figure 2) suggested nearly 80% of exogenous WR 1065 and WR 1729 disappeared from plasma within 10 minutes even at 4°C, while in a mixed monochloroacetic acid/perchloric acid solution both compounds were stable. WR 1065 and WR 1729 were quantitated by comparing their peak heights with the peak heights of the initial stability sample. In addition, varying degrees of plasma stability for both WR 1065 and WR 1729 could be achieved by acidification of the plasma samples (Figures 3 and 4). These results caused some alarm as the elapsed time, from the moment of blood withdrawal from the dog until acidification, was between 15 and 20 minutes. This meant the determined plasma values for WR 1065 could possibly represent only 15 to 20% of the mass present at the moment the blood was withdrawn. This uncertainty prompted modifications of the sample preparation procedure which reduced the entire process to no more than 2-3 minutes. Table V presents the WR 1065 plasma concentrations found in samples withdrawn at various times during dog dosing with ethiofos. Study No. 8 utilized the slower plasma sample preparation procedure while Study No. 10 employed the newer, more rapid scheme. The rapid sample preparation resulted in a decrease in the measured WR 1065 concentrations not the anticipated increase.

TABLE IV. ACCURACY OF WR 1065 ANALYTICAL METHOD

Spike Level µg/mL	Measured Level µg/mL	Percent Deviation (D)
2.5	2.2	-12.0
7.5	7.1	-5.3
35	38.3	9.4
80	81.9	2.4
125	120	-4.0
375	389	3.7

Average Deviation = $\frac{|D|}{n}$ = 6.1

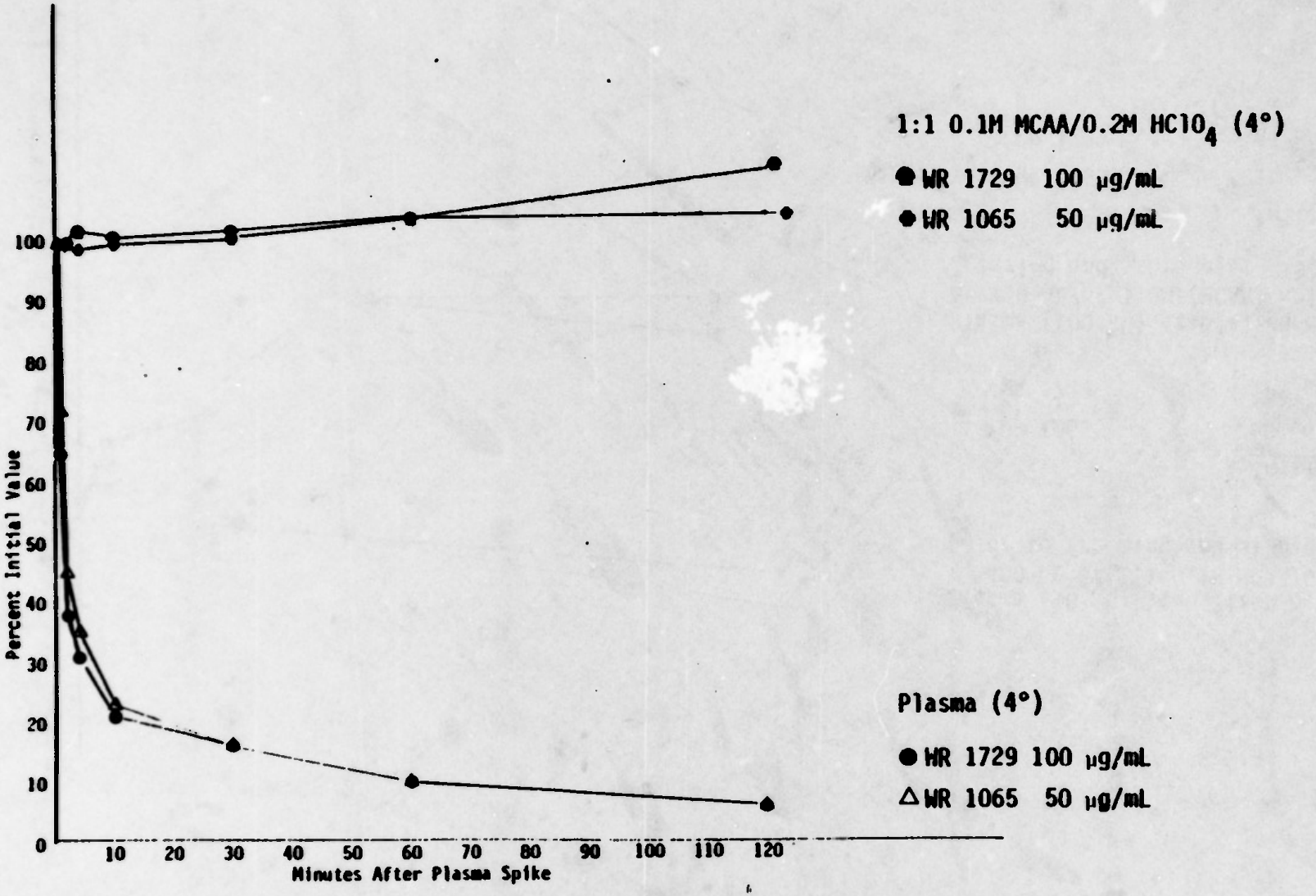
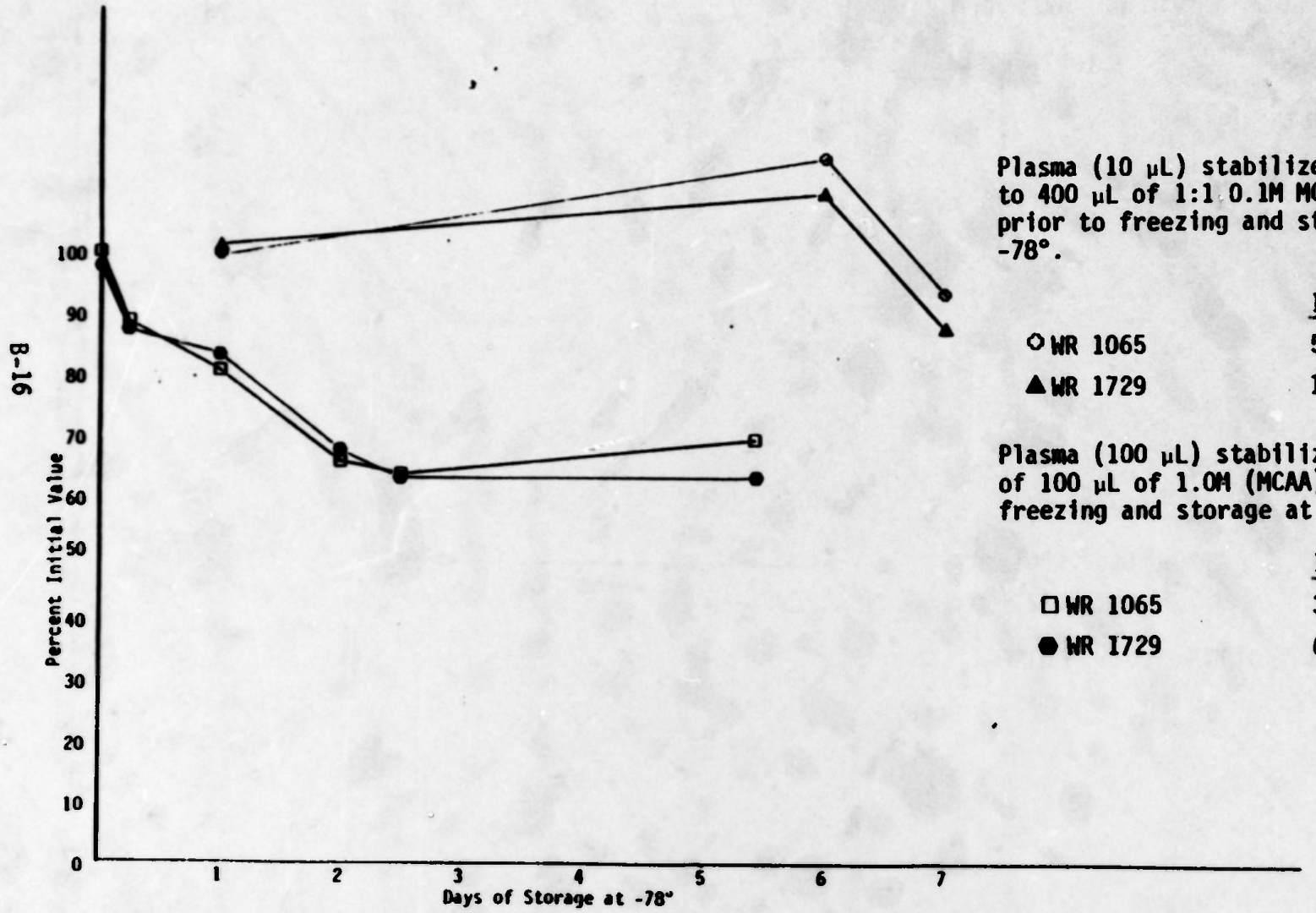


Figure 2. Stability of WR 1065 and WR 1729 in Plasma and 1:1 0.1M Monochloroacetic Acid (MCAA)/0.2M Perchloric Acid ($HClO_4$).



Plasma (10 µL) stabilized by addition to 400 µL of 1:1 0.1M MCAA/0.2M $HClO_4$ prior to freezing and storage at -78°.

	Initial Conc.
○ WR 1065	50 µg/mL
▲ WR 1729	100 µg/mL

Plasma (100 µL) stabilized by addition of 100 µL of 1.0M (MCAA) prior to freezing and storage at -78.

	Initial Conc.
□ WR 1065	33 µg/mL
● WR 1729	67 µg/mL

Figure 3. Stability of Plasma Solutions of WR 1065 and WR 1729 at -78° After Treatment with 1M MCAA or Mixed 0.1M MCAA/0.2M $HCLO_4$ 1:1.

Figure 4. Stability of WR 1065 and WR 1729 in Beagle Plasma Treated with 1:3 0.1 M Monochloroacetic Acid/0.2 M Perchloric Acid and Stored at -78°C.

TABLE V. WR 1065 PLASMA CONCENTRATIONS FOUND IN SAMPLES OBTAINED DURING ETHIOFOS INFUSION STUDIES

Time (Minutes)*	WR 1065 Concentration (µg/mL) Dog Dosing Study No. 8	Dog Dosing Study No. 10	
2 (A)	4.9	†	
4 (A)	15.5	†	
6 (A)	19.0	†	
8 (A)	31.5	†	
2 (P)	32.0	16.6	
4 (P)	31.0	14.7	14.2**
6 (P)	34.6	16.1	
10 (P)	-	22.6	
12 (P)	32.8	22.4	
15 (P)	38.2	22.0	22.0
22 (P)	34.9	21.6	
30 (P)	29.8	17.2	20.7
40 (P)	27.8	18.4	
50 (P)	23.3	15.7	
60 (P)	12.3	8.5	
70 (P)	9.7	6.0	
80 (P)	9.0	5.3	
90 (P)	6.1	4.5	
100 (P)	5.0	3.5	
110 (P)	3.5	3.2	
120 (P)	2.6	2.7	
180 (P)	1.2	1.3	
240 (P)	0.4	0.4	
300 (P)	0.4	0.33	
360 (P)	0.3	0.22	
480 (P)	0.2	0.22	
720 (P)	0.14	****	
960 (P)	***	****	
1440 (P)	***	****	
1800 (P)	***	****	
2800 (P)	***	****	
3240 (P)	***	****	
7200 (P)	***	****	
8640 (P)	***	****	

† No sample collected
*A Minutes into infusion
*P Minutes after end of infusion
** WR 1729 internal standard addition to whole blood
*** Value below sensitivity of assay, 0.1 µg/mL with 20 µL injection volume
**** Value below sensitivity of assay, 0.05 µg/mL with 100 µL injection volume

The feasibility of adding the internal standard directly to whole blood was also investigated. Three special blood samples drawn 4, 15, and 30 minutes post infusion were taken during Study No. 10. The blood volumes obtained varied, and the amount of WR 1729 solution added was double that added to an equivalent plasma volume. No visible change occurred upon internal standard addition. The tubes were inverted a few times to mix the components, centrifuged to separate the plasma and then treated in the usual manner. The WR 1065 concentrations determined using external calibration standards agreed with the values found for the normal samples following the procedure which involved using the internal standard (Table V). The internal standard could not be employed at this time with the whole blood samples as there was not available enough fresh whole blood to allow determinations of adequate calibration points.

Results from the two dosing studies using both plasma sample preparation schemes did not correlate with previous observations using fortified plasma (Figure 2). However, both sets of data would be consistent if the soluble plasma components had reactive sites specific for R-S-H compounds. If all available sites had reacted, the time elapsed during sample preparation would have only a minimal effect on the WR 1065 concentration, while addition of exogenous WR 1065 to plasma having soluble components which were reactive would result in rapid disappearance of WR 1065 as reaction occurred. To test this theory, fresh plasma was incubated for 1 hour at 4°C in the presence of 1000 µg/mL WR 1729. After 1 hour 100 µg/µL WR 1065 was added and the plasma concentration of WR 1065 followed over time. The results presented in Figure 5 show a gradual

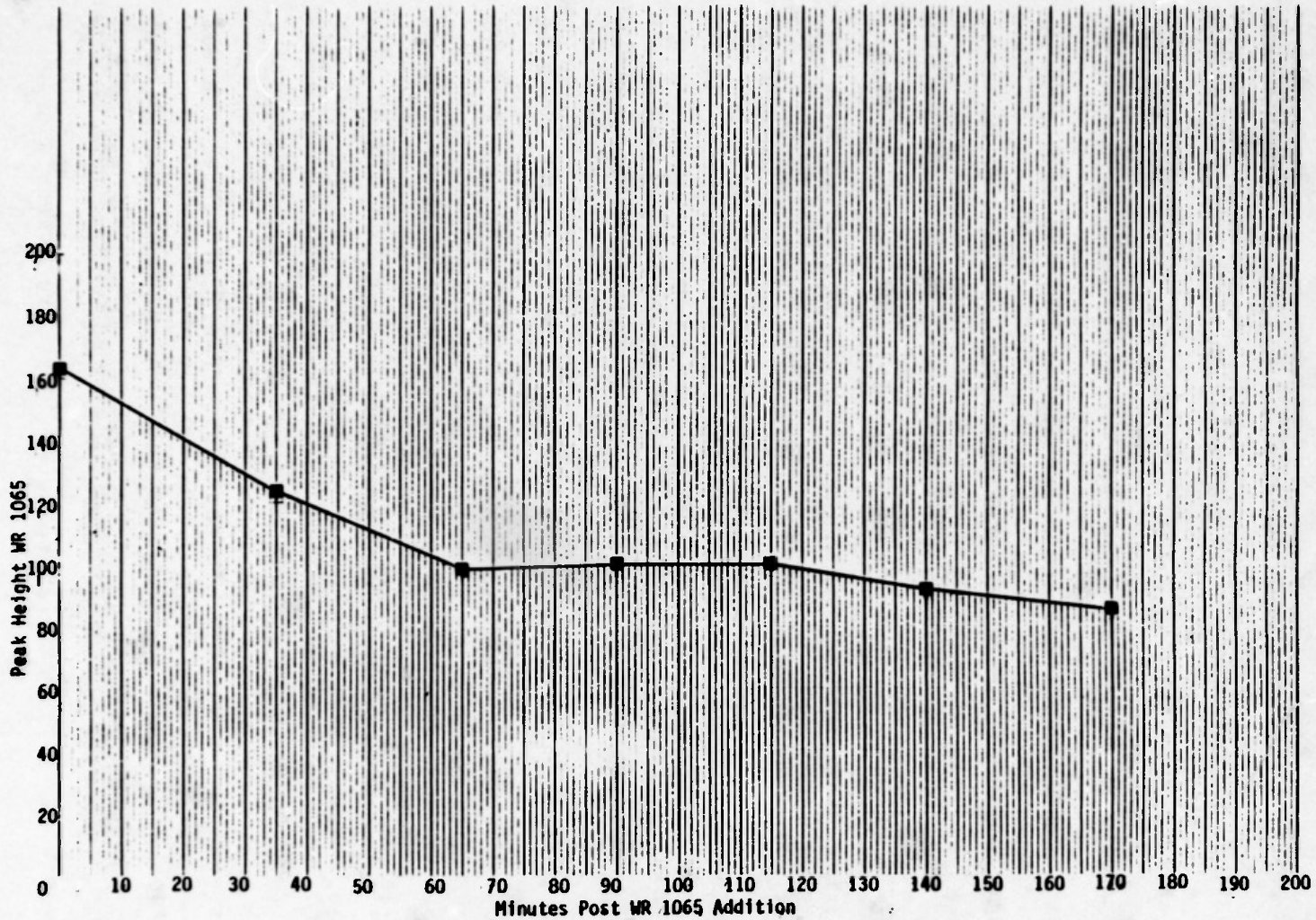


Figure 5. WR 1065 Peak Height Versus Minutes Post Addition to Plasma Pre-Incubated with 1000 µg/mL WR 1729

decrease in WR 1065 concentration which may be the result of exchange of WR 1729 for WR 1065 at the reactive sites. These observations suggest the soluble constituents of the plasma from the dosing studies may in fact be reacting with WR 1065 and what is measured is only unreacted WR 1065.

2. Ethiofos

Preliminary results from stability studies with ethiofos indicate its stability at 4°C in a mixed plasma monochloroacetic acid/perchloric acid solution adjusted to pH 3.0 (Figure 6). At a pH of 1.0 some decomposition occurred (Figure 7).

D. Increasing the Detection Limits

The increase in sensitivity necessary to quantify WR 1065 at the .05 µg/µL plasma level was achieved by changing injection volumes. Early work used a 20-µL injection loop. Increasing the loop size to 100 µL increased sensitivity without loss in resolution or increased interferences (Figure 8).

E. Biological Application

Table VI presents the WR 1065 concentration in the plasma samples obtained from ethiofos and WR 1065 infusion studies. The plasma drug level profile for all three studies are similar with the infusion study No. 9 showing the most rapid decrease in WR 1065 levels. This can partly be explained by the difference in dose levels [~11 millimoles (150 mg/kg) of ethiofos versus 5.8 millimoles (60 mg/kg) of WR 1065] but until more is known about clearance mechanisms, cellular distribution and binding, precise interpretation of the data is not possible.

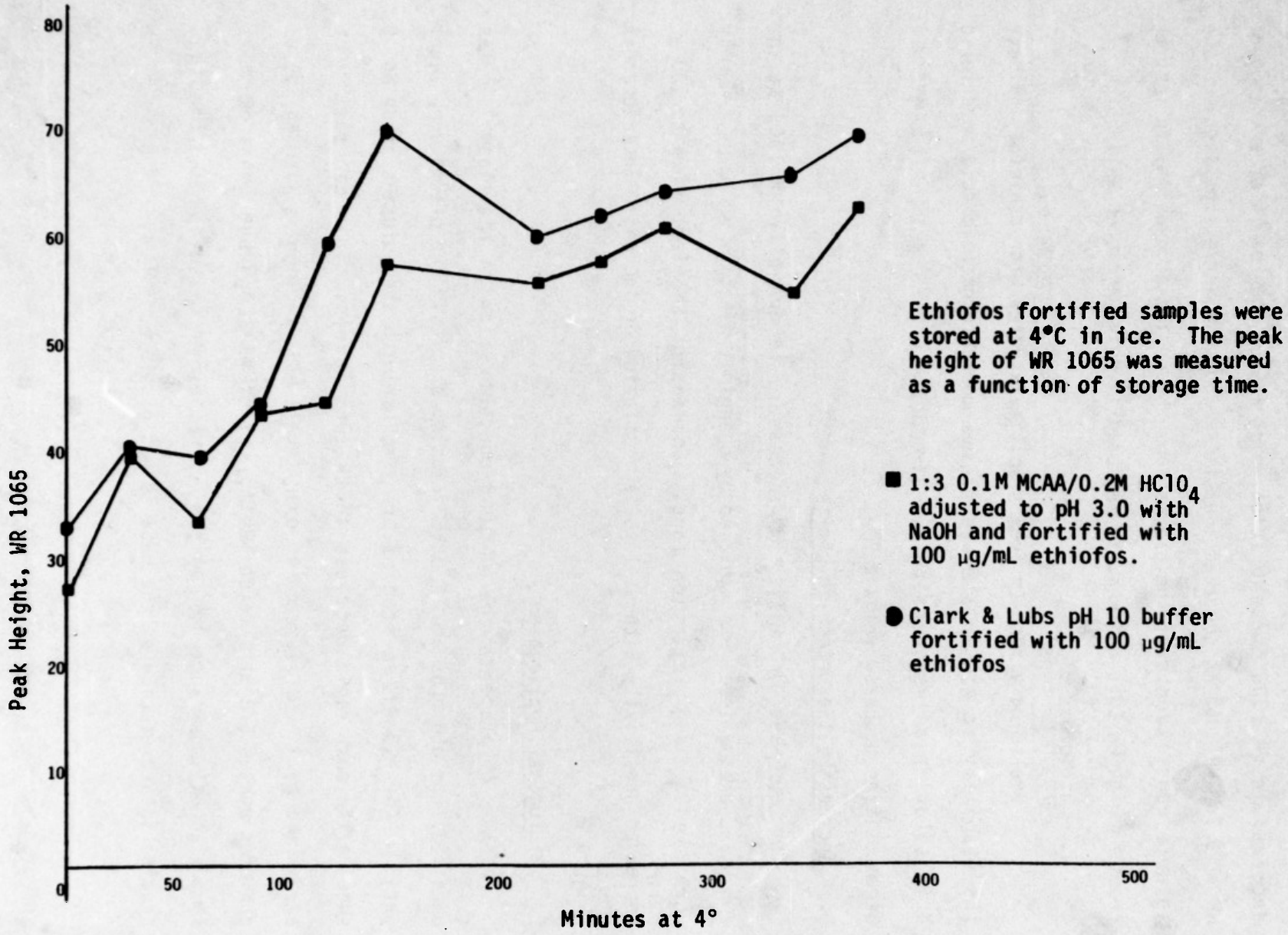
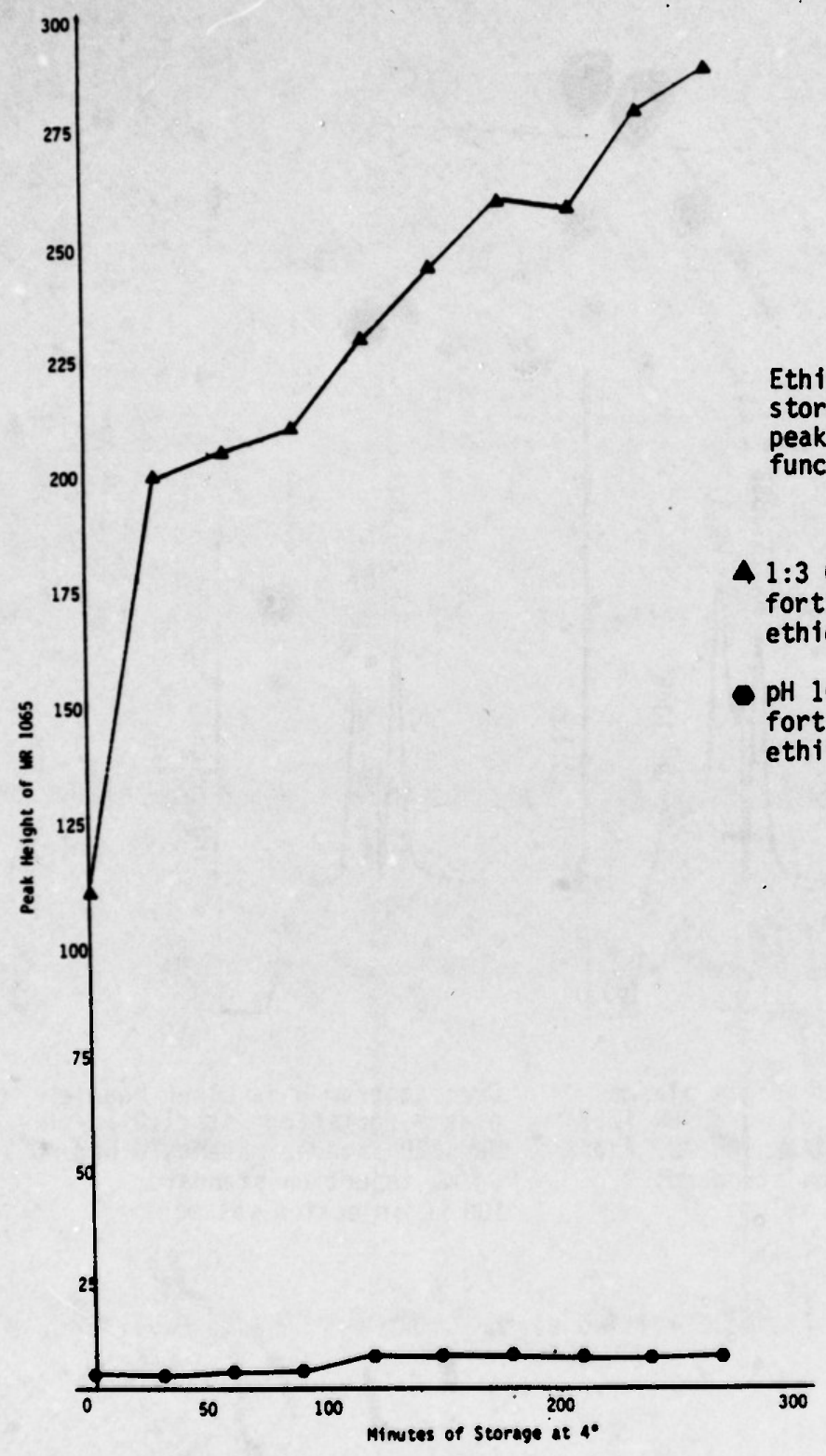


Figure 6. Stability of Ethiofos at pH 3.0.

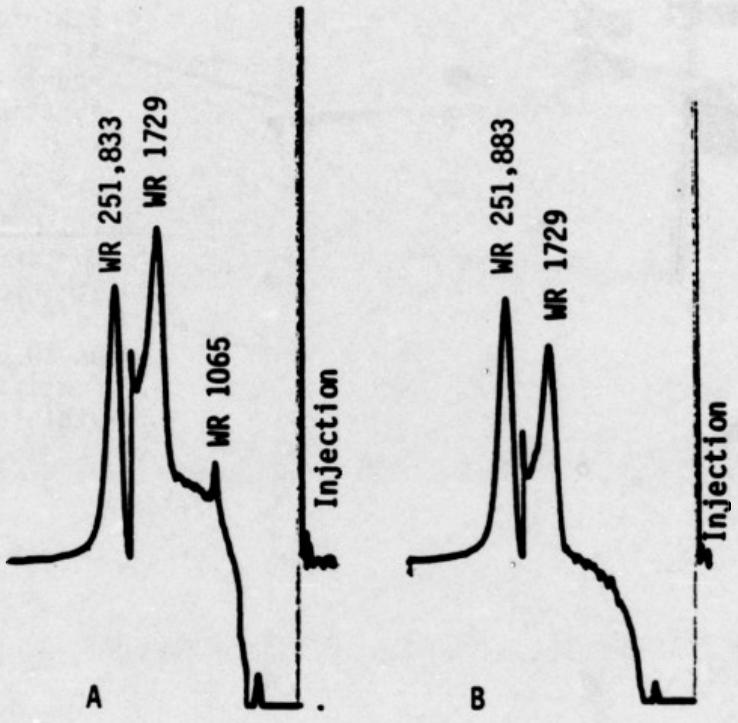


Ethiofos fortified samples were stored at 4° in ice. WR 1065 peak height was measured as a function of storage time.

▲ 1:3 0.1M MCAA/0.2M $HClO_4$ pH 1.0 fortified with 100 µg/mL ethiofos.

● pH 10 Clark & Lubs Control fortified with 100 µg/mL ethiofos.

Figure 7. Stability of Ethiofos at pH 1.0.



Chromatogram A is beagle plasma fortified with 0.05 µg/mL WR 1065 as well as 1.0 µg/mL WR 1729 and 40 µg/mL injection standard; 100 µL injection volume

Chromatogram B is blank beagle plasma fortified with 1.0 µg/mL WR 1729 internal standard and 40 µg/mL injection standard; 100 µL injection volume

Figure 8. Beagle Plasma Fortified at the 0.05 µg/mL Plasma Level with WR 1065

TABLE VI. WR 1065 PLASMA CONCENTRATIONS FOUND IN SAMPLES OBTAINED FROM ETHIOFOS AND WR 1065 INFUSION STUDIES

Time (Minutes)*	WR 1065 Concentration (µg/mL) Dog Dosing Study No. 8 (Ethiofos Infusion)	Dog Dosing Study No. 10 (Ethiofos Infusion)	Dog Dosing Study No. 9 (WR 1065 Infusion)
2 (A)	4.9	†	†
4 (A)	15.5	†	†
6 (A)	19.0	†	†
8 (A)	31.5	†	†
2 (P)	32.0	16.6	11.0
3 (P)	†	†	9.4
4 (P)	31.0	14.7	10.1
6 (P)	34.6	16.1	6.5
9 (P)	†		4.8
10 (P)	†	22.6	†
12 (P)	32.8	22.4	3.8
15 (P)	38.2	22.0	2.9
22 (P)	34.9	21.6	1.7
30 (P)	29.8	17.2	0.76
40 (P)	27.8	18.4	0.29
50 (P)	23.3	15.7	0.23
60 (P)	12.3	8.5	0.19
70 (P)	9.7	6.0	0.14
80 (P)	9.0	5.3	0.16
90 (P)	6.1	4.5	0.15
100 (P)	5.0	3.5	†
110 (P)	3.5	3.2	0.12
120 (P)	2.6	2.7	0.10
180 (P)	1.2	1.3	†
240 (P)	0.4	0.4	**
300 (P)	0.4	0.33	
360 (P)	0.3	0.22	
480 (P)	0.2	0.22	
720 (P)	0.14	**	
960 (P)	**	**	
1440 (P)	**	**	
1800 (P)	**	**	
2800 (P)	**	**	
3240 (P)	**	**	
7200 (P)	**	**	
8640 (P)	**	**	

† No sample collected
*A Minutes into infusion
*P Minutes after end of infusion
** Less than detection limit

F. Areas Under Active Investigation

There remain many aspects of the WR 1065 plasma assay which require further attention. Those areas currently under active investigation are:

- benefits of rapid sample preparation and internal standard addition;
- advantage of the addition of internal standard to whole blood before plasma separation;
- identification and suitability of compound for use as an injection standard, possibly $H_2N(CH_2)_4NH(CH_2)_3SH$ (WR 251833);
- stability of WR 1065 in preserved plasma;
- stability of ethiofos in plasma preserved for WR 1065 analysis;
- further definition of detector response at low (<5 µg/mL) WR 1065 plasma levels;
- quantification at levels <0.05 µg/mL plasma;
- precision and accuracy determinations with final analytical method;
- analysis of blind fortified samples to calculate method precision and accuracy.

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APPENDIX C

(HPLC Procedure for Assaying Ethiofos (WR 2721)

WR 2823-Containing Micro(Spheres/Capsules))

I. Sample Preparation

1. Weigh to nearest .0001 gm a 0.05 gm* microsphere (50 mg) sample(s) into a suitable containers <ie. 4 dram vial pre-rinsed with Milli-Q-HOH and Acetone. *If samples are microcapsules then weigh 75 mg.
2. Add 5 mL $CHCl_3$ (use pipette) to digest shell material and (use pipette) 5 mL pH 10 buffer to dissolve ethiofos or WR 2823.
3. Shake sample well, allow phases to separate by means of a centrifuge.* (Use centrifuge located in Metals Lab). Remove 1.0 mL (use pipette) of aqueous phase-upper layer and transfer to a 10 mL red volumetric flask. Dilute to mark with pH 10.0 buffer. *Centrifuge ~10-15 min.
4. Remove a 100 µL aliquot (place in plastic test tubes) and derivatize it by adding in succession:
    1) 300 µL pH 7.6 buffer - vortex 5-10 sec
    2) 200 µL Fluorescamine/Acetone Reagent - vortex 5-10 sec wait approximately one minute
    3) 200 µL Fluorescamine/Acetone Reagent - vortex
5. Remove ~50 µL and inject 20 µL into the HPLC column.
6. Anti-radiation compound will peak at ~4 min.

II. HPLC Conditions

Column:	Bio Rad RP 318 250 mm x 4.6 mm
Mobile Phase:	25% Acetonitrile HPLC 74% Milli-Q-HOH 1% 0.01 Molar TBAP
Flow Rate:	1.5 mL/min
Detection:	Waters Fluorescence 420 AC

III. Calibration

A solution of ethiofos or WR 2823 in the mobile phase is used as an external standard. Procedure used is described in sample preparation.

Pressure Limits: 3000 psi (Pump load)

Note: This procedure has been developed to assay microcapsules or microspheres containing a ethiofos or WR 2823 in a matrix of glycerides or fatty acids.

APPENDIX D

(Hydrolytic Stability of Ethiofos or WR 2823)

The method decribed below is based on that reported by the National Formulary XIII entitled, "Release Tablets and Capsules - In Vitro Test Procedure," with minor modifications.

I. Equipment

1. Rotating bottle apparatus with constant temperature bath (37°C).
2. Support glassware, screens and filters.

   90-mL bottles
   80-mL beakers
   4 dram vials
   Vacuum desiccator
   Stainless steel screen cloth, 105 μm
   Vacuum pump
   Analytical balance
   45-mL repeater pipettes

II. Test Solution

1. Gastric Fluid, Simulated (Buffer solution at pH level of 1).

   Buffer solutions are used as received. The solution used should have a pH of about 1.0. The pH of the buffer solution will be determined by pH meter before use.

III. Procedure

1. All weights and measurements shall be recorded.
2. There shall be two samples per test (1.5 hour).
3. For each formulation to be tested, weigh two samples (~50 mg microspheres or ~75 mg microcapsules) on the analytical balance and place in two 90-mL bottles marked with the sample number.
4. Add 45 mL buffered solution (specific pH) (37°C) to each bottle. Record time T(0) when the last bottle is filled. This is the starting time for the test.
5. Cap bottles tightly and rotate in 37°C bath at 10-12 RPM.
6. Remove the sample(s) at 1.5 hours from T(0). Separate the microspheres or microcapsules using a fine mesh stainless screen. The filtrates are discarded.

   Rinse the microspheres or microcapsules on the screen with water and place in a vacuum desiccator. Dry overnight at minimum pressure (vacuum pump). Place in clearly marked vials, flood with argon and retain in the freezer for analysis as described in HPLC Procedure for Assaying Ethiofos (WR 2721) or WR 2823)-Containing Micro(Spheres/Capsules).

APPENDIX E

(In Vitro Release Rate-Rotating Bottle Method A)

The method described below is based on that reported by the National Formulary XIII entitled, "Release Tablets and Capsules - In Vitro Test Procedure," with minor modifications.

I. Equipment

1. Rotating bottle apparatus with constant temperature bath (37°C).
2. Support glassware, screens and filters.

   90-mL bottles
   80-mL beakers
   4 dram vials
   Vacuum desiccator
   Stainless steel screen cloth, 105 μm
   Vacuum pump
   45-mL repeater pipette
   Analytical balance
   2000-mL volumetric flask

II. Synthetic Intestinal Solution

1. All weights and measurements shall be recorded.
2. Synthetic Intestinal Solution

   Mix: 13.6 g monobasic potassium phosphate
   76.0 mL 1N sodium hydroxide

   Dilute to 2000 mL with deionized water and adjust pH to 7.5 ± 0.1 with 1N NaOH if necessary. To each 100 mL solution to be used, add 1.0 g pancreatin just before use (this step is optional).

III. Procedure

1. All weights and measurements shall be recorded.
2. There shall be four sample times per formulation (at 1/4, 1/2, 1, and 2 hours).
3. For each formulation to be tested, weigh eight samples (approx. 50 mg microspheres or approx. 75 mg microcapsules) on the analytical balance and place in seven 90 mL bottles marked with the sample number and time interval.
4. Beginning with the 2-hour sample(s), add 45 mL intestinal fluid (37°C) to each bottle. Record time T(0) when the last bottle is filled. This is the starting time for the test.
5. Cap bottles tightly and rotate in 37°C bath at 12-12 RPM.

6. Remove the first sample(s) at 1/4 hour from T(0). Separate the microspheres or microcapsules using a fine mesh stainless screen. Place solutions (~3 mL is retained) in clearly marked 4 dram vials and retain for analysis. The microspheres or microcapsules on the screen with water and place in a vacuum desiccator under vacuum (utilizing a vacuum pump) to dry overnight. Place in clearly marked vials, flood with argon and retain in the freezer for analysis as described in HPLC Procedure for Assaying Ethiofos (WR 2721) or WR 2823-Containing Micro(Sphere/Capsules).

7. Repeat Step 6 at the proper time intervals for the remaining samples.

8. The solutions from Step 6 may be frozen if necessary until analysis is undertaken.

9. For assay of the synthetic intestinal fluid for ethiofos or WR 2823, approximtely 3 mL of the fluid is taken for the assay. The sample is analyzed using the ethiofos/WR 2723 procedure (beginning at Step 4) and utilizing an external standard of approximately 250 µg/mL ethiofos.

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REPLY TO
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31 JUL 1992

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Bldg. 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Development Command (USAMRDC), has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-80-C-0128. Request the limited distribution statement for AD Nos. ADB063672, ADB080528, ADB094912, ADB094846, [redacted], and ADB109038, be changed to [redacted] distribution unlimited," and that copies of these reports be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller, DSN 343-7325.

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